THE ROLES OF HISTONE DEACETYLASES 1 AND 2 IN HEPATOCELLULAR CARCINOMA

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THE ROLES OF HISTONE DEACETYLASES 1 AND 2 IN HEPATOCELLULAR CARCINOMA

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ABBREVIATIONS

Abl	V-abl Abelson murine leukemia viral oncogene homolog
AFP	alpha fetoprotein
AML	acute myeloid leukemia
APC	Adenomatous polyposis coli
ATM	ataxia telangiectasia mutated
Bax	Bcl-2 associated x protein
Bcl	B-cell lymphoma
Bcr	break point cluster region
BH3	Bcl-2 homology domain 3
Bid	BH3 interacting domain death agonist
Bmf	Bcl-2 modifying factor
BSA	bovine serum albumin
CBHA	m-carboxy cinnamic acid bishydroxamic acid
CCL	chronic lymphocytic leukemia
CDK	cyclin-dependent kinase
cDNA	complementary DNA
CK	casein kinase
CPS	counts per second
CRS	cutaneous radiation syndrome
CTCL	cutaneous T-cell lymphoma
CXCR	C-X-C motif receptor
DEPC	diethyl pyrocarbonate
DLBCL	diffuse large B-cell lymphoma

DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	Deoxyribonucleotide triphosphate
DR	death receptor
DTT	dithioreitol
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular signal-regulated kinase
FDA	Food and Drug Administration
FIH	factors inhibiting HIF
GADD	growth arrest and DNA damage
GSK-3	Glycogen synthase kinase 3
HAD	HDAC association domain
HAT	histone acetyltransferase
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDAC	histone deacetylase
HGF	hepatocyte growth factor
HHIP	Hedgehog-interacting protein
HIF	hypoxia inducible factor
HRP	horseradish peroxidase
IAP	inhibitor of apoptosis
IGF	insulin-like growth factor

IgG	immunoglobulin G
IPTG	isopropyl-beta-D-thiogalactoside
LB	lysogeny broth
LEF	Lymphoid enhancer-binding factor
MAPK	mitogen activated protein kinase
MBD	methyl CpG binding domain
MEF	mouse embryonic fibroblast
Met	mesenchymal-epithelial transition factor
MHC	major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B
miRNA	micro RNA
MMP	matric metalloproteinase
MMTV	mouse mammary tumor virus
mRNA	messenger RNA
MTA	metastasis-associated protein
mTOR	mammalian target of rapamycin
NAD+	nicotinamide adenine dinucleotide
NKG	natural killer cell protein group
NLS	nuclear localization signal
NSCLC	non-small cell lung carcinoma
PBS	phosphate buffered saline
PBST	phosphate buffered saline-Tween
PCR	polymerase chain reaction

PI	propidium iodide
PI3	phosphatidylinositol 3-kinase
PTEN	phsophatase and tensin homolog
pVHL	von Hippel-Lindau protein
Rb	retinoblastoma
RbAp	retinoblastoma-associated protein
RECK	reversion-inducing-cysteine-rich protein with kazal motifs
RNA	ribonucleic acid
ROS	reactive oxygen species
RPM	revolutions per minute
S1P	sphingosine-1-phosphate
SAHA	suberoylanilide hydroxamic acid
SDS	sodium deodecyl sulphate
SDS PAGE	SDS polyacrylamide gel electrophoresis
SHH	sonic hedgehog
siRNA	small interferring RNA
SIRT	sirtuin
SMAC	second mintochondria-derived activator of caspase
SMO	smoothened
SOC	super optimal broth
STAT	signal transducer and activator of transcription
TBE	tris-borate EDTA
TCF	T-cell factor
TGF	trasforming growth factor

TIMP	tissue inhibitor of metalloproteinase
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TSA	Trichostatin
VEGF	vascular endothelial growth factor
VPA	valproic acid

SUMMARY

Liver cancer is a disease that is more prevalent in Asia than the rest of the world. Of the various types of liver cancers, hepatocellular carcinoma (HCC) is the most common. The development of HCC is a multi-step process. During this process, the aberrant expression and activities of various genes contribute to the survival and proliferation of tumor cells. One family of proteins that is known to suppress the expression of tumor suppressor genes is histone deacetylase (HDAC). The inhibition of HDACs, by means of various classes of drugs collectively known as HDAC inhibitors, is currently being examined as a strategy to kill tumor cells.

In this study, we identified two members of the HDAC family to be highly expressed in human HCC tissue. Both HDAC1 and HDAC2 were upregulated in the HCC tumors compared to the matched non-tumor controls, and HDAC1 expression was found to be correlated with poor prognosis in the patients. When both HDAC1 and 2 were silenced in HCC cell lines, there was reduced colony formation, reduced proliferation, and increased apoptosis in the cells. These effects are attributed to the enzymatic activities of these 2 proteins, which have a compensatory effect on each other's expressions and activities. In addition, we also examined the change in gene expression profiles in HCC cells when HDAC1 and 2 were silenced individually and together, in comparison to the use of HDAC inhibitor PXD101.

Together, these results established the critical roles of HDAC1 and 2 in the survival and proliferation of HCC cells. We have also elicited their mechanism of actions by demonstrating the importance of their enzymatic activity as well as the compensatory effects on each other. Understanding these 2 members of the HDAC family would have significant impact on the design and use of HDAC inhibitors in the treatment of HCC.

CHAPTER 1

INTRODUCTION

CHAPTER 1 INTRODUCTION

1.1 Liver cancer

1.1.1 High occurrence and high mortality

Due to population aging and growth, cancer is fast becoming the leading cause of death. Liver cancer is the 5th most common cancer worldwide, with an alarming 748,300 new cases and 695,900 cancer deaths in 2008 (Jemal et al., 2011). The highest liver cancer rate is in East and Southeast Asia, with over half of the cases worldwide occurring in China alone. Between 1988 to 2001, the 5-year survival rate of liver cancer patient is only 8% in the United States and 5% in developing countries (Chuang et al., 2009).

1.1.2 Hepatocellular carcinoma (HCC)

There are many forms of liver cancers with different histological types. These include hepatocellular carcinoma, childhood hepatoblastoma, adult cholangiocarcinoma which originates from the intrahepatic biliary ducts, and angiosarcoma which originates from the intrahepatic blood vessels (Chuang et al., 2009). Of these, hepatocellular carcinoma (HCC) is the most common, accounting for 85% to 90% of all primary liver cancers (El-Serag and Rudolph, 2007). It frequently occurs in a liver with chronic hepatitis and cirrhosis, where many hepatocytes die and there is invasion by inflammatory cells and fibrosis (Thorgeirsson and Grisham, 2002).

1.2 Risk Factors for HCC

1.2.1 Hepatitis B and Hepatitis C viruses

The dominant risk factor for HCC is infection by Hepatitis B virus (HBV) or Hepatitis C virus (HCV). HBV infection is common in Asian countries excluding

Japan, which has more HCV-related cases. The virus can be transmitted from mother to child or via sexual intercourse. There is a 5- to 15-fold increased risk of HCC for HBV carriers compared to non-carriers (El-Serag and Rudolph, 2007).

The HBV is a double-stranded DNA containing virus that belongs to the family *Hepadnaviridae* (Sanyal et al., 2010). It can cause necroinflammation of liver cells, leading to cirrhosis. Hepatocytes will proliferate in order to regenerate the damaged liver. This high turnover in hepatocytes could result in accumulation of genetic mutations of the cells. Consequently, there will be increase in genetic changes, chromosome rearrangement, as well as activation and inactivation of oncogenes and tumor suppressor genes respectively (But et al., 2008). In the absence of cirrhosis, the HBV can also integrate itself into the host's genome, contributing to genomic instability (Szabo et al., 2004). Also, HBV produces HBx protein that is able to regulate expression of genes involved in cell proliferation, deregulate cell cycle control, and interfere with DNA repair and apoptosis (Feitelson, 1999).

The HCV is a RNA-containing virus that belongs to the *Hepacivirus* genus of the *Flaviviridae* family (Szabo et al., 2004). It is unable to integrate into the host genome, but its core protein can enter the host cell and localize on the mitochondrial membrane and endoplasmic reticulum. This promotes oxidative stress for the infected cell. Signaling pathways will be activated to upregulate genes involved in cytokine production and eventually inflammation, changes in apoptotic pathway and tumor formation (Sheikh et al., 2008).

1.2.2 Other risk factors

Other than HBV and HCV infection, aflatoxin contamination of food is also a major risk factor for HCC. It occurs commonly in Southeast Asia and China, where there is improper storage of food such as cereals and peanuts. Aflatoxin is a

mycotoxin produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* and is carcinogenic (Chuang et al., 2009). Aflatoxin B1 can cause p53 mutation by G:C to T:A transversions at the 3rd base in codon 249 of the gene (Greenblatt et al., 1994).
In addition, there is also evidence to suggest that alcohol drinking, smoking, obesity and diabetes as possible risk factors for HCC (Chuang et al., 2009).

1.3 Current treatment of HCC and problems

1.3.1 Diagnosis and staging

Diagnosis of HCC is generally made by radiological imaging and measuring serum Alpha Fetoprotein (AFP) level. If a liver mass is detected in a patient with chronic hepatitis or cirrhosis, there is a high likelihood of HCC. Biopsy may or may not be needed to proceed with assessment for treatment.

Tumor staging for HCC is done by tumor/node/metastasis (TNM) staging or Okuda staging system (Lau and Lai, 2008). The TNM system classifies tumors based on the size of the primary tumor (T), presence of lymph node metastasis (N), and distant metastasis (M), but does not look at liver function. On the other hand, Okuda staging system takes into account liver function such as presence of ascites, as well as albumin and bilirubin levels in the blood (Okuda et al., 1985).

1.3.2 Liver resection

The primary therapy for HCC is surgical resection of the liver. However, only 10% to 30% of HCC patients are suitable for surgery at the time of diagnosis (Lau and Lai, 2008). There are many criteria to satisfy before recommending surgery. Unsuitable patients include those with tumors that are too large resulting in insufficient hepatic remnant after surgery which may lead to subsequent liver failure, multifocal tumors that are too extensive, and distant metastasis (Lau, 1997).

The major problem with liver resection as treatment for HCC is tumor recurrence (Portolani et al., 2006). Recurrence could be due to either intrahepatic dissemination of the primary tumor or *de novo* tumor development. Intrahepatic dissemination is usually the cause, evident from the fact that the presence of satellite nodules and microvascular invasion are the 2 main predictors for tumor recurrence (Adachi et al., 1995; Nagasue et al., 1993). Such recurrence commonly takes place within 3 years after surgery, and is characterized by multifocal and aggressive tumor (Imamura et al., 2003). Repeated hepatectomy can be done to treat recurrent disease with a 5-year survival of up to 50%, but re-recurrence rate is generally high (Itamoto et al., 2007).

1.3.3 Liver transplantation

Orthotopic liver transplantation is the best therapy for HCC, provided there is no macroscopic vascular invasion and metastasis. Not only does it remove the tumor burden, it also treats the underlying liver disease that could lead to recurrence in the patient. However, there are very limited number of organs available for transplant, leading to prolonged waiting time which is associated with high dropout rates as the disease progresses beyond selection criteria for transplant (Rahbari et al., 2011). Living donor liver transplantation can increase the pool of available donors. Nevertheless, there are many ethical issues to be considered given the donor morbidity of up to 40% and mortality of 0.5% (Trotter et al., 2002). In addition, there is a need for immunosuppression in patients receiving liver transplant. Two of such immunosuppressive drugs, Cyclosporine and Tacrolimus, have raised controversy for their use in HCC patients as they have been shown to have potential tumor-promoting effects (Guba et al., 2004).

1.3.4 Radiation therapy

External beam radiation therapy is seldom used in HCC due to the low tolerance of the non-tumorous portion of the liver. It takes 120 Gy to kill the tumor cells in HCC while liver irradiation beyond 40 Gy can cause radiation-induced liver disease (Lawrence et al., 1995). Therefore, selective intra-arterial radiotherapy (SIRT) is used to deliver radioactive microspheres to the tumor internally. However, SIRT can also cause complications such as postembolic syndrome, characterized by fatigue, abdominal pain, and fever (Rahbari et al., 2011).

1.3.5 Chemotherapy

Systemic chemotherapy is used to treat patients with unresectable HCC. Doxorubicin is widely used in these patients but the response rate is very low (less than 20%) with no survival advantage (Lai et al., 1988). Other drugs such as Tamoxifen and Somatostatin have also been tested in clinical trials but results are not satisfactory. However, there has been some success in the clinical trial of the drug Sorafenib in recent years. Sorafenib is an oral multikinase inhibitor that can block cell proliferation and neoangiogionesis (Wilhelm et al., 2008). In a multicenter phase III clinical trial on Sorafenib to treat 602 advanced HCC patients, the treatment group demonstrated 31% reduction in the risk of death and a longer median survival of 10.6 months compared to 7.9 months in the placebo group (Llovet et al., 2008). The time to progression (TTP) based on independent radiological review was 5.5 months for patients treated with Sorafenib and 2.8 months for the control group. However, like most chemotherapy drugs, there were adverse side effects associated with the use of Sorafenib. These include diarrhea, fatigue, weight loss, and hand-foot skin reaction. Although there was no death related with toxicity being described, there was drug discontinuation in 15% of the patients due to the adverse effects.

1.4 Molecular mechanisms of HCC development

Just like many other types of cancer, the development of HCC is a multi-step process. Vogelstein proposed that there must be at least 3 genomic hits for solid tumor such as HCC to develop (Vogelstein and Kinzler, 2004). The risk factors mentioned in previous sections can set the stage for hepatocarcinogenesis by causing the initial damage to the liver. Additional genomic hits, in the form of mutations or epigenetic regulation, can alter key genes in the cancer pathways, thus inducing the cell to acquire malignant phenotype.

According to Hanahan and Weinberg, pathways disrupted in cancer can be divided into 6 groups based on their functions: evading apoptosis, unlimited replicative potential, self-sufficiency in growth signals, insensitivity to anti-growth signals, angiogenesis, and tumor invasion and metastasis (Hanahan and Weinberg, 2000). Numerous pathways are affected due to molecular changes in hepatocarcinogenesis.

1.4.1 Pathway involved in cell survival

The 2 major pathways that are responsible for HCC cell survival are the Wnt and Hedgehog (Hh) signaling pathways.

Upon binding of the Wnt ligand to the membrane receptor Frizzled, a cascade of events occurs. The axin/GSK-3/APC complex which normally promotes the degradation of beta catenin in the cytoplasm will be inhibited, allowing beta-catenin to now enter the nucleus to interact with the TCF/LEF family transcription factors. This leads to the transcription of various oncogenes, such as c-myc, cyclin D, and survivin, which are involved in cell survival.

Two important genes in the hedgehog signaling pathway, Sonic Hedgehog (SHH) and smoothened (SMO), are found to be overexpressed in many cases of HCC. This leads to the activation of the pathway (Lachenmayer et al., 2010). On the other hand, a negative regulator of the pathway Hedgehog-interacting protein (HHIP) is downregulated in many HCC cases by methylation and/or loss of heterozygosity.

1.4.2 Pathways involved in cell proliferation

The pathways contributing to HCC cell proliferation are mesenchymal-epithelial transition factor (c-Met), insulin-like growth factor (IGF), Ras-mitogene activated protein kinase (Ras-MAPK), and PI3/Akt/mTOR, pathways.

Hepatocyte growth factor (HGF) can activate the c-Met pathway which is responsible for invasive growth in cancer, angiogenesis, proliferation and migration (Villanueva et al., 2007). In HCC, upregulation of HGF in cirrhotic liver and c-Met amplification and mutation has been reported.

In addition, the IGF pathway is also frequently activated in HCC (Villanueva et al., 2007). The upregulation of IGF-1 and IGF-2 and silencing of IGF binding proteins can lead to proliferation, as well as anti-apoptotic and invasive phenotype of the cell. IGF signaling can also activate the downstream Ras-MAPK pathway.

The PI3/Akt/mTOR pathway is involved in numerous cellular processes such as proliferation, cell cycle progression, tumor growth, angiogenesis, apoptosis and cell differentiation. In HCC, poor prognosis has been associated with activated Akt, which can be activated by IGF signaling (Schmitz et al., 2008). Mammalian target of rapamycin (mTOR) can sense nutritional status and allow progression from G1 to S phase of the cell cycle (Sabatini, 2006). Aberrant mTOR signaling is commonly found in HCC.

1.4.3 Apoptotic pathways

Apoptosis, or programmed cell death, is a mechanism by which a cell dies after sustaining damage, with minimal disruption to its neighboring cells. One characteristic of many cancer cells is their ability to evade apoptosis. The activation of apoptosis is either by intrinsic stimulus such as DNA damage, or by extrinsic signals such as the binding of pro-apoptotic factors to the cell surface. The downregulation of pro-apoptotic factors (p53, Fas, PTEN, Bax, Bid) and the upregulation of many anti-apoptotic factors (beta-catenin, Akt, RaS/ERKs, HGF, EGFR ligands, c-IAP1, NF-kappaB, Snail) are observed in HCC (Fabregat et al., 2007).

1.5 Epigenetic regulation in cancer

There are many genes involved in the above mentioned pathways that are essential in hepatocarcinogenesis. The expression of these genes can be altered by genomic changes such as DNA mutation, deletion, amplifications and translocations. However, classical genetics alone cannot be used to explain how identical twins can have different phenotypes and susceptibilities to diseases (Esteller, 2008). Alternatively, gene expression can be regulated by epigenetic mechanisms. The term “epigenetics” was first used by Conrad Waddington about 70 years ago and its definition has evolved over time (Waddington, 1939). One of the most accepted definition described epigenetics as heritable changes in gene expression without changes in DNA sequences (Jones and Baylin, 2007). These include histone modification, DNA methylation, and microRNA expression.

1.5.1 DNA methylation

The human genome contains about 2-5% CpG dinucleotides (a cytosine next to a guanine) which mostly consists of repetitive sequences (Sincic and Herceg, 2011).

The CpG islands are located near the transcriptional start site in the promoter regions of many genes. DNA methylation is a process by which DNA methyltransferase (DNMT) transfers a methyl group to the carbon 5 position of the cytosine ring of the CpG dinucleotide covalently (Kanai, 2010). In normal cells, DNA methylation patterns are tissue-specific and gene specific. It is important in epigenetic reprogramming during development (Mann and Bartolomei, 2002). In cancer cells, there is dysregulation of DNA methylation, with hypermethylation at the gene promoter (CpG island specific) region as well as global hypomethylation (Sincic and Herceg, 2011). The degree of methylation at the promoter region can impact gene expression by affecting the binding of transcription factors and methyl-binding proteins. The hypermethylation at the promoter results in the silencing of numerous tumor-suppressor genes as well as other cancer-associated genes, such as *RB, VHL* and *E-cadherin* (Kanai, 2010). The list of genes whose promoters are hypermethylated in cancer has been rapidly growing over the years. While hypermethylation at promoter region has been well-studied, much less is known about the global hypomethylation observed in cancer cell. It has been proposed that genes that are normally repressed in a healthy cells may be re-expressed in a cancer cell due to the loss of methylcytosine as observed in hypomethylation (Sincic and Herceg, 2011). These includes proto-oncogenes and imprinted genes, as well as viral transposons that contribute to genomic instability (Esteller, 2008). In HCC, DNMT1 mRNA expression is higher in liver tissue with chronic hepatitis or cirrhosis than that in normal livers, and even higher in HCC cases (Saito et al., 2001; Sun et al., 1997). The overexpression of DNMT1 in HCCs is also correlated with more poorly differentiated tumor types (Saito et al., 2003). In addition, Kondo et al. studied the methylation status of 8 CpG islands in non-cancerous and cancerous liver tissues and

found that it is higher in cancerous liver tissue (Kondo et al., 2000). In another study, the CpG methylation profile of HCC was done in a number of cancer-related promoters. By correlating the data with clinical outcomes in the patients, it was found that the methylation signature can be used to predict survival and clinical parameters such as grade and stage (Hernandez-Vargas et al., 2010). Taken together, DNA methylation is a mechanism by which molecular dsyregulation of genes occurs in HCC and has useful prognostic applications.

1.5.2 MicroRNA

MicroRNAs (miRNAs) are 22-nucleotide non-coding RNA that can bind to the 3' untranslated region of their target mRNA in a sequence-specific manner, leading to mRNA degradation or translation inhibition (He and Hannon, 2004). The miRNA expression profiles was found to be different between normal and tumor, as well as among tumor types (Calin and Croce, 2006). In HCC cell line, the let-7 family of miRNA was shown to inhibit expression of Bcl-xL and enhanced sorafenib-induced apoptosis (Shimizu et al., 2010). There are at least 25 aberrantly expressed miRNA and their respective targets identified in HCC (Huang and He, 2011).

1.5.3 Histone modification

In eukaryotes, DNA is coiled around 4 histone units (H2A, H2B, H3, and H4) to form nucleosomes. Other than their role as packaging material for DNA, histones are also involved in gene regulation, DNA damage repair, replication, and recombination (Lennartsson and Ekwall, 2009). The "tails" of the histones protrude out from the surface of the chromatin polymer, and can undergo posttranslational modification such as acetylation, ubiquitylation, sumolytion, phosphorylation, and methylation. Based on the histone code hypothesis, the distinct modification of the histone tails can act sequentially or in combination to form the "histone code" which

is read by other proteins to cause downstream biological events (Strahl and Allis, 2000). Each histone unit has many modification sites subjected to different types of modifications: H2A contains 13 sites, H2B contains 12 sites, H3 contains 21 sites, and H4 contains 14 sites (Zhang et al., 2003). The modification at one site can also influence that at another. Therefore, the number of possible combination and permutation of the histone code is enormous. The histone code can be stable, making it inheritable from one cell generation to the next. It can also be transient, making it dynamic and subjected to changes depending on environmental signal and the cell's physiological state.

1.5.3.1 Histone acetylation and deacetylation

Of the various types of histone modifications, histone acetylation is the most common and well-studied. Acetylation can neutralize the positive charge of the N-termini of the histone lysine residues, thus reducing their affinity for DNA so that the histone can be displaced from the nucleosome, which will then unfold and allow access by transcriptional factors (Lee et al., 1993). In other words, the chromatin is in a more "relaxed" or opened state when the histone tail lysine residues are acetylated. This is generally associated with gene activation. On the other hand, deacetylation increases the ionic interaction between the negatively charged DNA and the positively charged histones, leading to condensed chromatin structure and gene silencing. The acetylation level is due to the balance of activities by 2 types of enzymes: the histone acetyltransferases (HATs) and histone deacetylases (HDACs). Various cancers have been shown to have altered HAT and HDAC activities (Timmermann et al., 2001).

1.6 Histone acetyltransferases (HATs)

There are 2 types of HATs: the A-type HATs which are nuclear and transcription related; and B-type HATs which are involved in acetylation of histones (Grunstein, 1997). Instead of binding directly to DNA, HATs are recruited to the promoter by transcription factors (Roth et al., 2001). Acetylation of lysine on histone tails is not a random event. HATs can have preference for one site over another. Also, HATs can interact with other HATs and transcription co-repressors and co-activators to form a functional protein complex (Marks et al., 2001). Other than histones, HATs can also target non-histones proteins known as factor acetyltransferases (FATs), such as p53 and E2F (Roth et al., 2001). The acetylation of these proteins will affect their DNA binding property and their functions as transcription factors.

In various hematological and epithelial cancers, genes that encodes for HATs such as p300 and CBP, are found to be mutated, translocated, amplified, or overexpressed (Marks et al., 2001). For example, missense mutation of *p300* has been found in gastric and colorectal cancers (Giles et al., 1998). In HCC, the loss of heterozygosity around the *CBP* locus has been reported (Sakai et al., 1992).

1.7 Histone deacetylase (HDAC)

1.7.1 HDAC family of proteins in mammals

HDAC enzymes were first discovered in yeast. There are 18 mammalian HDACs identified so far. They are classified into classes based on their homology to that in yeast (Ropero and Esteller, 2007). Class I HDAC, which includes HDAC 1, 2, 3, and 8, are homologous to Rpd3 in yeast. They are ubiquitously expressed in the nucleus of many human tissues and cell lines. Class II HDACs are homologous to Hda1 and are subdivided into class IIa (HDAC 4, 7, and 9) and IIb (HDAC 6 and 10).

Their expression is tissue-specific, and can translocate between the cytoplasm and nucleus. Their primary substrates are non-histone proteins so they are more appropriately called lysine deacetylases (Marks and Xu, 2009). Class III HDACs, also known as sirtuins (SIRT1-7) are homologous to the yeast Sir2 family. They are dependent on coenzyme NAD+ for them to be active. Class IV has only one member HDAC11, which shares conserved residues with class I and II HDACs.

1.7.2 HDACs can function in a protein complex

HDAC do not directly bind to DNA. Instead, they are recruited to specific chromosome regions by transcription and chromatin-related factors to form large multiunit protein complexes (Yang and Seto, 2003). For example, a core complex consisting of HDAC1, HDAC2, and the histone chaperones retinoblastoma-associated proteins (RbAp) 48 and 46 can interact with SAP30 and Sin3 to form the Sin3 corepressor complex, which can interact with sequence-specific DNA binding proteins to repress specific genes (Laherty et al., 1997). The same core complex can also interact with Methyl CpG binding domain 3 (MBD3), Metastasis-associated protein 2 (MTA2), and the ATP-dependent chromatin-remodeling protein Mi2 to form the NuRD corepressor complex, which has a more global effect on transcription repression but can also bind to gene-specific transcription factors (Verdin, 2006). Therefore, HDACs can exert different effects through different binding partners.

1.7.3 Regulation of transcription by HDACs

1.7.3.1 Gene silencing

As mentioned in the previous section, deacetylation of histone tails can limit DNA accessibility to the transcriptional activators as well as promote association of silencer, thus cause gene silencing. Other than exerting its effect at the chromatin level, HDACs can directly target the transcription machinery by deacetylating TAF_I68

to inhibit Polymerase1-dependent transcription (Muth et al., 2001). Also, while acetylation of transcriptional activators can affect their DNA-binding ability, stability, activation potential, nuclear localization and coactivator interaction, deacetylation can reverse these effects (Yang and Seto, 2003). It has been demonstrated that the class III HDAC SIRT1 can deacetylate p53 to inhibit its DNA-binding and transcriptional activation activity, thereby blocking its function in cellular senescence and apoptosis (Langley et al., 2002; Luo et al., 2001).

1.7.3.2 Gene activation

Despite the conventional mechanism of HDAC repressing gene expression, there has been evidence that HDAC is involved in gene activation. A member of the class I HDAC in yeast, Hos2, has been shown to be required for efficient gene transcription (Wang et al., 2002). In addition, HDAC activity was needed for the Mouse mammary tumor virus (MMTV) promoter to function (Lee et al., 2011b). It was proposed that the recruitment of Polymerase II requires the deacetylation of the proteins that are part of the reinitiation scaffold, and inhibition of HDAC would impair this recruitment, leading to decreased rate of transcriptional initiation.

1.7.4 Regulation of HDACs

There are post-translational modifications that regulate HDACs functions. Inhibition of phosphatase can disrupt HDAC1 and HDAC2 complexes by increasing their phosphorylation (Galasinski et al., 2002; Pflum et al., 2001). On the other hand, these HDAC complexes are stabilized by specific phosphorylation by casein kinase 2 (CK2) (Tsai and Seto, 2002). These apparent contradicting effects of phosphorylation on HDAC functions may imply that the effect is site-specific. In addition, sumoylation of HDAC1 is needed for its cell-cycle arrest and apoptotic response (David et al., 2002). Other than post-translational modification, it was recently

discovered that lipid sphingosine-1-phosphate (S1P) can bind to and inhibit the activity of both HDAC1 and HDAC2 (Hait et al., 2009). Also, recombinant HDACs produced *in vitro* are inactive, implying that co-factors such as Rb and MTA2 are necessary for their activation and function *in vivo* (Guenther et al., 2001).

1.8 HDAC1 and 2

1.8.1 Phylogenetic ancestry

Both HDAC1 and HDAC2 belong to class I HDACs and are highly homologous. They share 83% amino acids identity (Yang and Seto, 2008). Phylogenetic analysis have shown that HDAC1 and HDAC2 genes originated from a single ancestor after a gene duplication event (Gregoretti et al., 2004). Gene duplication usually results in functional diversification when one of the 2 paralogs acquires new function or tissue-specific distribution. However, based on the strong sequence constraint between HDAC1 and 2, and that this gene duplication leading to HDAC1 and 2 was relatively recent, it was predicted that little functional divergence has taken place (Gregoretti et al., 2004). While this may be true in some biological processes, there is also evidence to suggest distinct functions of HDAC1 and 2 and they will be discussed in subsequent sections.

1.8.2 Structure

The HDAC1 and 2 proteins contain several domains. The largest and most important of these is the N-terminal catalytic domain which consists over 300 amino acids. The active site on the catalytic domain is a pocket with 2 adjacent histidine residues, 2 aspartic acid residues, and 1 tyrosine residue to form a "charge-relay" system with an essential Zn^{2+} ion (Brunmeir et al., 2009). When the Zn^{2+} ion is displaced from the pocket, such as by HDAC inhibitors, the charge-relay system

cannot function. There is also the N-terminal HDAC association domain (HAD) which is important for homo- and heterodimerization (Taplick et al., 2001), and the C-terminal IACEE domain that is important to binding with pRb (Brehm et al., 1998).

In addition to these domains that are common between HDAC1 and HDAC2, there are those that are found uniquely on HDAC1 or HDAC2. There is a nuclear localization signal (NLS) at the C-terminal of HDAC1 that is not found in HDAC2 (Taplick et al., 2001). There is also a previously unrecognized region at the C-terminal of HDAC2 predicted to have high propensity for coiled-coil (Gregoretti et al., 2004). This may imply that HDAC2 can have protein-protein interaction with unique partners to execute differential functions from HDAC1.

1.8.3 Functions in normal cells development

HDAC1 is essential for embryonic development. Knocking out both HDAC1 alleles in mice was embryonic lethal before E10.5, due to proliferation defects and retarded development (Lagger et al., 2002). Aberrant development was observed as early as E7.5. In these HDAC1-deficient mice, there was significant reduction in deacetylase activity in the Sin3 and NuRD complexes, as well as increase in levels of cyclin-dependent kinase inhibitors p21 and p27 in the embryonic stem cells.

On the other hand, the effect of knocking out HDAC2 in mice is not as straightforward. Montgomery et al. found that HDAC2 knockout mice can survive until the perinatal period but die shortly after, due to multiple cardiac defects (Montgomery et al., 2007). These defects include loss of the right ventricle lumen of the heart, with thickened interventricular septum as well as increased apoptosis. However, when a conditional knockout was done to delete HDAC2 specifically in the heart, the mice were able to survive to adulthood without gross cardiac abnormality. Another group did not observe lethality in HDAC2 knockout mice despite its

involvement in cardiac function (Trivedi et al., 2007). Interestingly, one group found reduction in cell number as well as the thickness of the intestinal mucosa, and a body weight reduction of up to 40% in HDAC2-null mice (Zimmermann et al., 2007).
In addition to its role in cardiac development, HDAC2 was also found to be involved in regulating memory formation and synaptic plasticity (Guan et al., 2009).

1.9 Cooperative and distinct functions of HDAC1 and 2

1.9.1 Redundancy of HDAC1 and HDAC2 functions

With a high homology between HDAC1 and 2 and their co-existence in the same protein complexes, one would expect some redundancy in their functions. Several experiments suggest that HDAC1 and 2 may compensate for the function of the other.

Firstly, despite the cardiac abnormality observed in HDAC2-null mice, Montgomery's group did not find any abnormality after knocking out HDAC2 specifically in the heart (Montgomery et al., 2007). It was only when a double deletion of both HDAC1 and HDAC2 was done in the heart that the knockout mice displayed postnatal lethality at day 14 with increased apoptosis in the heart. Secondly, it was demonstrated that while ablation of either HDAC1 or HDAC2 in mouse embryonic fibroblast (MEF) did not have any overt phenotype under normal growth condition, a double knockout MEF resulted in growth arrest and senescence (Wilting et al., 2010). The cell cycle analysis of these MEF showed that both HDAC1 and 2 are needed for G1 to S phase transition. Deletion of both would lead to senescent-like G1 arrest. Thirdly, there is compensation mechanism between HDAC1 and HDAC2 when one of them is being perturbed. When either HDAC1 or HDAC2 was depleted, the protein level of the other was found to be increased in murine tissues and cell lines

(Lagger et al., 2002; Senese et al., 2007). This change was observed in the protein level but not mRNA, suggesting that this reciprocal regulation may be occurring at the translational or post-translational level, possibly by modulating protein stability or protein-protein interaction.

1.9.2 Distinct functions of HDAC1 and HDAC2

Despite of their high homology, HDAC1 and 2 are not completely redundant. They have specific and different functions which cannot be replaced by the other. For example, overexpression of HDAC2 but not HDAC1 in neurons can reduce dendritic spine density and synapse number and plasticity (Guan et al., 2009). Also, knockdown of HDAC2, but not HDAC1, increased p27 in rat renal interstitial fibroblast NRK49F (Pang et al., 2011). Similarly, HDAC2, but not HDAC1, can inhibit proliferation and induce senescence in the breast cancer MCF7 cells (Harms and Chen, 2007). The same group also demonstrated that HDAC2 can modulate the ability of p53 to bind DNA, thus controlling the transcriptions of p53-dependent genes. On the other hand, the loss of HDAC1, but not HDAC2, can affect embryonic stem cells differentiation as HDAC1-deficient cells formed smaller embryoid bodies with preferential differentiation toward mesodermal and ectodermal lineages (Dovey et al., 2010).

In fact, HDAC1 and 2 can have opposing effects. In mouse liver cells AML12, while silencing of HDAC1 can suppress TGFbeta1-induced apoptosis, silencing of HDAC2 increased spontaneous apoptosis and enhanced transforming growth factor (TGF)beta1-induced apoptosis (Lei et al., 2010). This reciprocal effect on cell viability by HDAC1 and 2 is mediated through their differential regulation of extracellular regulated kinase (ERK)1/2.

1.10 Inhibition of HDAC

In recent years, HDAC inhibition was recognized as a therapeutic strategy to treat various diseases by reversing the aberrant epigenetic state. These include treatment of neurodegenerative diseases (Selvi et al., 2010), acute pancreatitis (Escobar et al., 2010), and rheumatoid arthritis (Chung et al., 2003). Due to the effectiveness of HDAC inhibitors in killing tumor cells over normal cells, they have been most widely used in the treatment of various cancers.

HDAC inhibitors include various classes of hydroxamic acids, electrophilic ketones, benzamides, cyclic peptides, short chain fatty acids, boronic acid-based compounds, benzofuranone and sulfonamide containing molecules (Marks, 2010b). Many of them have similar structural characteristics, such as the zinc-binding moiety in the catalytic pocket, opposite capping group and a straight chain alkyl, vinyl, or aryl linker that connects the two. HDAC inhibitors work by having these functional groups interact with the relatively conserved regions of HDAC (Finnin et al., 1999)

1.11 Biological effects and mechanisms of action of HDAC inhibitors

There are many biological effects of HDAC inhibitors that make them effective therapeutic agents against cancer.

1.11.1 Apoptosis

1.11.1.1 Intrinsic pathway

Numerous studies demonstrated the involvement of the intrinsic or mitochondrial apoptotic pathway in HDAC inhibitor-induced cell death. For example, the overexpression of the anti-apoptotic proteins B-cell lymphoma-2 (BCL-2) and B-cell lymphoma-extra large (BCL-XL), both of which are essential in the mitochondrial pathway, blocked suberoylanilide hydroxamic acid (SAHA)-induced

apoptosis *in vitro* (Vrana et al., 1999). Similarly, *in vivo* studies using a syngeneic mouse model of Burkitt's lymphoma showed that primary B-cell lymphoma that overexpressed BCL-2 was resistant to SAHA, suggesting that activation of the mitochondrial pathway is required (Bolden et al., 2006).

It is not fully understood how HDAC inhibitors activate the mitochondrial apoptotic pathway. One possibility is that they change the balance in the expression of pro-apoptotic and anti-apoptotic proteins, or there can also be activation of proteins or pathways upstream of the mitochondrial pathway. Examples of such proteins are the BH3-only proteins Bid, Bim, and Bmf. Bid was shown to be cleaved and activated upon HDAC inhibition (Ruefli et al., 2001). Bim was upregulated transcriptionally after treatment by SAHA and TSA and promoted apoptosis (Zhao et al., 2005). Bmf was transcriptionally activated by HDAC inhibitors depsipeptide and m-carboxy cinnamic acid bishydroxamic acid (CBHA), while knocking Bmf down can block mitochondrial membrane damage and partly rescue clonogenic potential of cells treated by these HDAC inhibitors (Zhang et al., 2006).

In addition, the regulation of reactive oxygen species (ROS) production or activity can also mediate HDAC inhibition-induced activation of the mitochondrial apoptotic pathway. ROS is a natural byproduct of normal oxygen metabolism and play important roles in cell signaling. Their level can increase in the presence of environmental stress. HDAC inhibitor can promote the accumulation of ROS in tumor cells while treatment of free-radical scavengers can reduce the HDAC inhibition-induced apoptosis (Ruefli et al., 2001). Interesting, ROS production can also transcriptionally induce and activate Bim, linking it to the involvement of the BH3-only protein (Sade and Sarin, 2004).

1.11.1.2 Extrinsic pathway

Other than the intrinsic pathway, HDAC inhibition can also cause cell death via the extrinsic (death-receptor) apoptotic pathway. Upon HDAC inhibitor treatment, many tumor necrosis factor (TNF) receptor superfamily members and their ligands, such as TNF-related apoptosis-inducing ligand (TRAIL), death receptor 5 (DR5), Fas, and TNF-alpha were found to be transcriptionally activated (Johnstone, 2002). Blocking the death-receptor signaling pathway can abrogate HDAC inhibitor-induced apoptosis. For example, in an *in vivo* study using the PML-RAR transgenic mice that develop acute myeloid leukemia (AML), it was shown that the suppression of TRAIL and Fas using siRNA can reduce valproic acid (VPA)-induced apoptosis by 50% (Insinga et al., 2005).

1.11.2 Growth arrest

Most of the HDAC inhibitors, except tubacin, can induce cell cycle arrest at the G1 to S phase boundary (Haggarty et al., 2003). This is mediated by the retinoblastoma protein (pRb) and related proteins. Treatment with HDAC inhibitors leads to p53-independent induction of *CDKN1A* which encodes for p21 protein that promotes hypophosphorylation of pRb leading to cell cycle arrest (Richon et al., 2000). Also, HDAC inhibition-induced repression of cyclin A and cyclin D contributes to the loss of CDK 4 and CDK2 kinase activities as well as hypophosphorylation of pRb (Sandor et al., 2000). In addition, HDAC inhibition transcriptionally represses CTP synthase and thymidylate synthetase which are involved in DNA synthesis (Glaser et al., 2003). The direct effect of chromatin remodeling and the subsequent changes in gene expression can induce cell-cycle regulatory genes such as GADD45 and cause the upregulation of TGFbeta receptor

signaling which represses c-MYC, leading to cell cycle arrest (Chen et al., 2002; Jaboin et al., 2002).

Other than arresting cells at the G1/S phase, HDAC inhibition can mediate G2/M-phase arrest by activation of the G2-phase checkpoint. This, however, is not as common as the G1 arrest. It was proposed that while low concentration of HDAC inhibitors induces G1 arrest, higher concentration can induce both G1 and G2/M arrest (Richon et al., 2000). It is not fully understood how HDAC inhibition leads to G2 arrest. One possible mechanism could be the hyperacetylation of pericentric heterochromatin and the loss of the checkpoint would cause abnormal chromosomal segregation and nuclear fragmentation (Taddei et al., 2005). Because most tumor cells have defective G2 checkpoint, the HDAC inhibitor treated cells would accumulate in the G2/M phase and eventually pass this G2 checkpoint to undergo apoptosis (Peart et al., 2003). On the other hand, normal cells with a functional G2 checkpoint would be resistant to HDAC inhibition-induced apoptosis (Johnstone, 2002). This explains the differential effects of HDAC inhibitors on normal and tumor cells. Such tumor-selective killing of HDAC inhibitors makes them a favorable strategy in cancer therapy.

1.11.3 Mitotic disruption and autophagy

HDAC inhibition can cause mitotic defects due to aberrant histone acetylation in the heterochromatin and centromere domains (Xu et al., 2007). Histone acetylation can interfere with histone phosphorylation, thereby disrupting the function of mitotic spindle checkpoint proteins (Dowling et al., 2005). This results in transient arrest at prometaphase, and eventually aberrant mitosis such as missegregation and loss of chromosomes occurs (Qiu et al., 2000). In colon cancer cell lines, HDAC inhibitor can induce polyploidy and mitotic defects, leading to senescence (Xu et al., 2005).

In an experimental model using cervical cancer cell line HeLa, it was demonstrated that HDAC inhibitors can still induce cell death even when caspase activation was blocked by overexpression of Bcl-XL (Shao et al., 2004). Instead of undergoing apoptosis, these cells showed morphology typical of autophagic cell death.

1.11.4 Anti-angiogenesis, anti-metastasis and invasion

Angiogenesis is a process that involves the growth of new blood vessels to supply the metabolic needs of the growing tumor. Hypoxia inducible factors (HIF) are transcription factors for angiogenic genes (Brown and Wilson, 2004). Under hypoxic conditions, class I HDAC1, 2, and 3 are all activated in transformed cells, thus downregulating p53 and von Hippel–Lindau protein (pVHL), resulting in downregulation of factors inhibiting HIF (FIH) (Liang et al., 2006). This leads to activation of HIF-1alpha and angiogenesis. HDAC inhibition can prevent this tumor angiogenesis by inhibiting the HIF. Several HDAC inhibitors, such as TSA, vorinostat, FK228, butyrate and LAQ824 have been found to repress angiogenesis *in vitro* and *in vivo*, and downregulate pro-angiogenesis factors such as HIF-1alpha and vascular endothelial growth factor (VEGF) (Deroanne et al., 2002). In addition, HDAC inhibition was demonstrated to reduce expression of the chemokine (C-X-C motif) receptor 4 (CXCR4) (Crazzolara et al., 2002). This would prevent the homing of bone-marrow progenitor and circulating endothelial cells to the site of angiogenesis.

Other than exerting its effects on the primary tumor, HDAC inhibition can also affect metastasis. Metastasis is the growth of tumor distant from the site of the primary tumor. It is a multi-step process that enables the tumor cells to overcome barriers to local invasion, intravasation, survive in circulation, extravasation and eventually outgrowth to produce marcrometastases at a distant site. Many proteins are involved in facilitating this process. For example, matrix metalloproteinases (MMPs)

can degrade the extracellular matrix to allow invasion and intravasation. HDAC inhibitors can transcriptionally repress MMPs such as MMP2 and MMP9 (Klisovic et al., 2003). Concurrently, there is upregulation of Reversion-inducing-cysteine-rich protein with kazal motifs (RECK) which is known to negatively regulate MMP, as well as upregulation of tissue inhibitor of metalloproteinase (TIMP) (Liu et al., 2003). In addition to these *in vitro* studies, there are *in vivo* studies to demonstrate the anti-metastatic effect of HDAC inhibition. For example, using intrasplenic implant in mice model, treatment by HDAC inhibitor HA-butyrate can reduce incidence of metastasis and prolong survival (Coradini et al., 2004).

1.11.5 Anti-tumor immunity

There is a growing pool of evidence to show that HDAC inhibition can increase anti-tumor immunity by making the tumor cells more attractive immune targets, influencing immune cells activities, or by altering the production of cytokines (Bolden et al., 2006).

HDAC inhibition can upregulate major histocompatibility complex (MHC) class I and II proteins, and co-stimulatory/adhesion molecules and intracellular adhesion molecule 1 (ICAM1) (Maeda et al., 2000; Magner et al., 2000). In addition, HDAC inhibition induces MHC class I chain-related molecules MICA and MICB on the tumor cell surface. These molecules bind to the activating immunoreceptor natural killer cell protein group (NKG)2D on the cell surface of natural killer cells, γδ T cells and CD8 T cells. Tumor cells that express MICA and MICB on their surface are targets for NKG2D-restricted cytotoxicity. Upon treatment by HDAC inhibitor, HCC cells but not normal hepatocytes, have upregulated MICA and MICB and were killed by natural killer cells (Armeanu et al., 2005).

Signal transducer and activator of transcription 1 (STAT1), STAT3, and nuclear factor-κB (NF-κB) are considered as "master immune regulatory transcription factors" and their gene regulatory activities are regulated by acetylation (Chen et al., 2001; Nusinzon and Horvath, 2003; Yuan et al., 2005). It is possible that changes in cytokine profiles and their effects on immune cell functions after HDAC inhibition may be mediated through these transcriptional factors.

1.12 HDAC inhibitors in cancer therapy

1.12.1 Clinical trials

There are currently many HDAC inhibitors that are undergoing various phases of clinical trials for different types of cancer (Table 1.1)

Table 1.1 HDAC inhibitors in clinical trials (adapted from Marks, 2010 and Federico, 2011)

HDAC Inhibitor	Other common identifiers	Clinical trial phase
Valprioc Acid	Depakene	Phase I/II in hematological malignancies and solid tumors; FDA-approved for epilepsy, seizures, bipolar disorder
Vorinostat	Suberoylanilide hydroxamic acid (SAHA), Zolinza	Phase I/II in hematological malignancies and solid tumors; FDA-approved for CTCL
Panobinostat	LBH589	Phase I/II in hematological malignancies and solid tumor
Belinostat	PXD101	Phase I/II in hematological malignancies and solid tumor
Entinostat	MS-275	Phase I/II in hematological malignancies and solid tumor
MGD0103	Mocetinostat	Phase I/II in hematological malignancies and solid tumor
Romidepsin	Depsipeptide, Istodax, FK228	Phase I/II in hematological malignancies and solid tumor, FDA-approved for CTCL
Givinostat	ITF-2357	Phase I/II in hematological malignancies and solid tumor
PCI-24781	CRA-2024781	Phase I
Phenylbutyrate	VP-101, EI-532	Phase II

Of these, Vorinostat (SAHA) was approved by the United States of America Food and Drug Administration (FDA) in 2006 for the treatment of refractory cutaneous T-cell lymphoma (CTCL) (Marks and Breslow, 2007). A multicenter phase IIb trial tested the drug on 74 patients with CTCL who had at least 2 prior therapies. They were given daily dose of 400mg of Vorinostat orally and have an overall response rate of 29.7%, a 6.1 months median duration of response, and a 9.8 months median time to progression (Olsen et al., 2007). The side effects included diarrhea, fatigue, nausea, thrombocytopenia and anemia. Vorinostat was also tested in other malignancies such as relapsed diffuse large B-cell lymphoma (DLBCL), acute myeloid leukemia (AML), platinum-resistant epithelial ovarian cancer, primary peritoneal carcinoma, and non-small cell lung carcinoma (NSCLC) (Crump et al., 2008; Ramalingam et al., 2007).

Another drug approved by the FDA is Romidepsin (desipeptide) and it is also for treatment of CTCL (Federico and Bagella, 2011). Romidepsin is a natural compound isolated from *Chromobacterium violaceum*. It was first tested for its antibacterial activity but was found to exhibit cytotoxicity on tumor cells. The main targets of this drug are class I HDACs. The 2 phase II multicenter clinical trials recruited a total of 167 patients with refractory CTCL. The median time to response was 2 months with an overall response rate of about 34%. Side effects of the drug include nausea, fatigue, vomiting, anorexia, and cardiotoxicity. Other than CTCL, desipeptide was also tested in AML, chronic lymphocytic leukemia (CLL), lung cancer, hormone refractory prostate cancer, and renal cancer (Byrd et al., 2005; Fouladi et al., 2006; Schrump et al., 2008).

1.12.2 Synergism with other anti-cancer treatments

1.12.2.1 Drugs

In addition to being used as a single agent to treat various types of cancers, HDAC inhibitors have also shown synergism with other drugs in killing tumor cells. These include proteasome inhibitor bortezomib, Bcr-Abl kinase inhibitor, imatinib, cyclin-dependent kinase inhibitor flavopiridol, IkBa phosphorylation inhibitor BAY 11-7082 and many others (Jagannath et al., 2010; Marks, 2010a).

The rationale behind the choice of drug combination is usually based on the molecular mechanisms by which the drugs work. For example, tumor suppressor genes are most effectively reactivated by sequential DNA demethylation followed by histone acetylation (Cameron et al., 1999). This provided the basis for the use of demethylating agent such as 5-aza-cytidine with HDAC inhibitors. This treatment regime was tested clinically for patients with myelodysplastic syndrome and AML. Partial and complete responses have been reported with reactivation of genes such as *CDKN1A* (encoding p21 protein) (Gore et al., 2006).

HDAC inhibitors can also be used together with other conventional chemotherapeutic drugs to augment their effects. For example, a standard drug for relapsing myeloma, dexamethasone, was shown to be synergistic with the HDAC inhibitor LAQ824 due to enhanced apoptotic signaling (Catley et al., 2003; Chauhan et al., 2001). Dexamethasone induces the release of second mitochondria-derived activator of caspases (SMAC) from the mitochondria into the cytosol, leading to neutralization of the inhibitory effects of inhibitor of apoptosis (IAPs) on caspase 9. This enhances the caspase 9-mediated cytotoxic effects of LAQ824. Concurrently, LAQ824 activates caspase 8 which serves as an additional apoptotic signal to that induced by dexamethasone.

1.12.2.2 Radiation

Many HDAC inhibitors have been shown to act synergistically with γ-irradiation to kill tumor cells *in vitro* (Kim et al., 2004; Zhang et al., 2004). There were also further studies in animal models to show similar results. For example, using the DU145 prostate carcinoma xenograft, a combination of radiation and HDAC inhibitor MS-275 resulted in histone hyperacetylation and greater inhibition of tumor growth compared to single treatment (Camphausen et al., 2004). Similarly, HDAC inhibitor Valproic acid can radiosensitize the human brain tumor cell line U251 in mice xenograft model, resulting in delayed tumor growth (Camphausen et al., 2005). Ataxia telangiectasia mutated (ATM) is a serine/threonine protein kinase activated by double-strand break damage to DNA, and can activate downstream targets to mediate biological response to DNA damage such as apoptosis (Shiloh, 2003). HDAC inhibition can activate ATM even in the absence of DNA double-strand breaks (Bakkenist and Kastan, 2003). Moreover, HDAC inhibition can suppress DNA repair with downregulated expression of DNA repair proteins Ku70, Ku86 and DNA protein kinase (Munshi et al., 2005). The activation of ATM as well as the suppression of DNA repair may explain how HDAC inhibition acts synergistically with radiation therapy to kill tumor cells.

In addition, HDAC inhibitors were shown to decrease cutaneous radiation syndrome (CRS), which is skin-morbidity caused by radiation. HDAC inhibitors, such as Valproic acid, can promote healing of radiation-induced wounds and decrease skin fibrosis (Chung et al., 2004). Therefore, HDAC inhibitors greatly increase the therapeutic gain in cancer radiotherapy by enhancing the inhibition of tumor growth and protection of normal tissues.

CHAPTER 2

AIMS

CHAPTER 2 AIMS

Histone deacetylase (HDAC) inhibitors are gaining ground as a new class of chemotherapeutic agent in recent years. Much effort has been put into the development of new HDAC inhibitors that are more isoforms-specific, in hope of reducing off-target effects associated with drug used. To effectively design and utilize HDAC inhibitors in the treatment of cancer, we need to better understand the functions of the HDAC isoforms and their mechanism of actions. Numerous studies tried to test the effect of knocking down each individual isoform of HDAC on gene expressions and survival in cancer cells. However, the results vary between different tissues and contexts. We seek to study the expression, functions, and mechanism of action of 2 highly homologous histone deacetylases, HDAC1 and 2, in hepatocellular carcinoma (HCC).

The first step to understanding the role of a protein in the development and progression of cancer is to compare its expression in the tumor to that in the normal tissue. To establish the clinical significance of HDAC1 and 2 in HCC, we will study the expression of HDAC1 and HDAC2 in human HCC samples and correlate their expression with clinicopathological parameters such as patient survival. This will establish the clinical significance of HDAC1 and 2 in HCC. Secondly, we will study the individual and cooperative roles of HDAC1 and 2 in HCC cell lines. We will focus specifically on cell survival and proliferation, which are critical characteristics of tumor cells that chemotherapy targets. This would be done by measuring the ability of the cells to form colonies, as well as the cell cycle profiles. Thirdly, the mechanism by which HDAC1 and 2 exert their effects on the cells would be examined. We will test if the functions of HDAC1 and 2 are dependent on their enzymatic activity. Lastly,

we will study the gene targets of HDAC1 and 2 and validate their roles in mediating the effects of HDAC1 and 2 in cell survival and proliferation.

This study would shed light on the roles of the 2 important members of the HDAC family in HCC, to help in the understanding of their mechanism of action.

CHAPTER 3

MATERIALS &

METHODS

CHAPTER 3 MATERIALS & METHODS

3.1 Tissue Microarray

3.1.1 Tissue Samples

A total of 358 hepatocellular carcinoma samples (179 sets of tumor and paired non-tumor) were included in this study. These were obtained from the Department of Pathology, National University Hospital of Singapore. There was no selection bias regarding gender, age, clinical presentation or tumor staging. Morphologically representative area of the tumor was annotated by the pathologist and 1.5mm tissue cylinders were punched from the donor tissue block and deposited into a recipient block using the Advanced Tissue Arrayer (Chemicon International, USA). The recipient tissue block was cut using a microtome into sections and placed onto a coated glass slide for immunohistochemical staining.

3.1.2 Immunohistochemistry

Tissue sections were deparaffinized in xylene and rehydrated in serial alcohol dilutions. Antigen retrieval was performed by heating the sections in Antigen Unmasking Solution (Vector Laboratory, USA) for 20 min using the microwave oven. The sections were then treated with 3% H_2O_2 to remove endogenous peroxidase activity, washed in PBST, and incubated with primary antibodies overnight at 4°C with gentle shaking. After optimization was performed to obtain optimal staining intensity, rabbit polyclonal antibody against HDAC1 from Abcam was used at 1:8000 dilution, while rabbit polyclonal antibody against HDAC2 from Santa Cruz was used at 1:200 dilution. Rabbit IgG (non-immune serum) was used as negative control.

After the sections were washed 3 times in PBST for 5 mins each, they were incubated with secondary antibody, which is a goat anti-rabbit IgG conjugated with avidin-biotinylated horseradish peroxidase (DAKO, Glostrup, Denmark). Lastly, the sections were washed 3 times in PBST for 5 mins each and incubated for 1 min with DAB substrate. Counterstaining was done using Hematoxylin solution (Sigma) and the sections were then dehydrated in serial alcohol dilution and mounted with coverslips.

3.1.3 Scoring of Tissue Microarray

Each sample on the tissue microarray slides was scored based on the intensity of staining in the nuclei of the hepatocytes. Whenever staining was observed, it was almost always limited to the nucleus and not the cytoplasm. A score of 0 indicated no nuclear staining while a score of 1, 2 and 3 represented low, moderate, and intense nuclear staining respectively. To obtain an index to whether the HDAC expression is upregulated, the score of the non-tumor sample was subtracted from that of the matched tumor sample (T-N). A positive index (T-N>0) would indicate that HDAC expression was upregulated in the tumor for that sample pair, a negative index (T-N<0) would indicate that HDAC expression was downregulated in the tumor for that sample pair, while an index of 0 would indicate that HDAC expression was not changed for that sample pair.

3.1.4 Statistical analysis

Statistical analysis was performed using SPSS. Survival rates of patients with different HDAC1 and HDAC2 expression levels were analyzed using Kaplan-Meier analysis and log-rank test. Statistical significance was accepted as a P value of less than 0.05.

3.2 Cell lines and cell culture

3.2.1 Cell lines

The human colon cancer cell lines, HCT116 and HT29, human hepatocarcinoma cell lines, HEP3B, HEPG2 and PLC5, were purchased from American Type Culture Collection (Rockville, MD). The HCT116 p53-/- cells were a kind gift from Dr. Vogelstein's lab at Johns Hopkins University (Baltimore, MD). The HCT116 and HT29 cells were cultured in McCoy's 5A (Sigma) medium supplemented with 10% fetal bovine serum (Gibco). The HEP3B, HEPG2 and PLC5 cells were cultured in DMEM (Sigma) medium supplemented with 10% fetal bovine serum (Gibco). The cells were maintained in an incubator at 37°C in a 5% CO_2 humidified atmosphere.

3.2.2 Transient transfection

Twenty-four hours before transfection, the cells were counted using a hemocytometer and plated at a density such that they would be about 40% confluent on the day of transfection.

For the overexpression of plasmid, 1μg of plasmid DNA or empty vector control was diluted in 125μl of Opti-MEM medium (Gibco), which was added to 5μl of Lipofectamine2000 Reagent (Invitrogen) that had been diluted in 125μl of Opti-MEM medium. The mixture was incubated at room temperature for 20-30 min before it was added dropwise to the cells plated in a 6-well plate with 750μl of Opti-MEM medium. The plate was incubated at 37°C in a 5% CO_2 humidified atmosphere for 4-5 hours before the Opti-MEM medium was replaced with the usual media in which the cells were normally cultured.

For the knockdown of genes, 20pmol of siRNA (Invitrogen) or universal control siRNA with matching %GC content was diluted in 250μl of Opti-MEM

medium (Gibco), which was added to 3µl of Lipofectamine RNAiMax Reagent (Invitrogen) that had been diluted in 250µl of Opti-MEM medium. The mixture was incubated at room temperature for 10-20 min before it was added dropwise to the cells plated in a 6-well plate with 500µl of Opti-MEM medium. The plate was incubated at 37°C in a 5% CO_2 humidified atmosphere for 4-5 hours before the Opti-MEM medium was replaced with the usual media in which the cells were normally cultured.

3.3 Western Blot

3.3.1 Protein extraction

Cells were washed in 1X PBS and collected by trypsinization and pelleted at 1000 RPM for 5 min at 4°C. The cell pellet was washed in cold 1X PBS and stored in -80°C until protein extraction.

Total protein extraction was performed by adding 50-100µl lysis buffer (6M urea, 1% 2-mercaptoethanol, 50mM Tris buffer pH 7.4, 1% SDS in PBS pH 7.4) to the cell pellet. To lyse the cells, sonication was done at 20kHz for 3 pulses of 15 sec each with the 2mm microtip probe using the High Intensity Ultrasonic Processor 130W model (SciMed).

Frozen normal liver was obtained from Department of Pathology, National University of Singapore. It was taken from a patient who has colorectal cancer with liver metastases and was undergoing surgery. The frozen tissue was weighed, homogenized with 3 volume of lysis buffer in a glass homogenizer, and sonicated as described above. After centrifugation at 13,000 RPM for 15 min at 4^0C, the supernatant was collected as protein lysate.

3.3.2 Protein quantification

The samples were quantified using the BioRad Protein Assay Kit (BioRad) with bovine serum albumin (Sigma) as the standard. The reagent was diluted 5X with distilled water before use. Each sample was serially diluted between 20X to 40X and 10μl of each diluted sample was added to a well in a 96well plate, in duplicates. Two hundred μl of diluted reagent was added to each well and incubated at room temperature for 5 min before reading the absorbance at 595nm using the spectrophotometer. The protein concentration would be calculated using the standard curve generated by the BSA standards of known concentrations.

3.3.3 SDS PAGE and transfer

Ten to 20μg of each sample was mixed with 5X loading buffer (10% SDS, 50% glycerol, 0.01% bromophenol blue, 7% DTT, 50mM Tris pH 6.8), heated at 95°C for 4 min and ran in a 4% stacking/10-12% resolving polyacrylamide gel in Glycine running buffer (0.1% SDS, 14.4mg/ml Glycine, 3.03mg/ml Tris) at 130V for 1 hour. A prestained protein ladder (BioRad) was run alongside as a marker for the molecular weight. After electrophoresis, the gel was equilibrated in transfer buffer (20% ethanol, 0.1% SDS, 14.4mg/ml Glycine, 3.03mg/ml Tris) for 15 min before being set up for transfer onto nitrocellulose membrane (Hybond C-Extra, 0.45 μm, Amersham Biosciences) at 100V for 1.5 hours.

3.3.4 Immunodetection

Nitrocellulose membrane was blocked in 5% non-fat milk (Anlene) in PBST (1X PBS, 0.1% Tween-20) for 1 hour at room temperature on a shaker. After blocking, the membrane was incubated in primary antibody diluted in 3% non-fat milk in PBST for 1 to 2 hours at room temperature. The membrane was then washed in PBST for 5 min thrice, and incubated with the corresponding secondary antibody

(anti-mouse, or anti-rabbit, or anti-goat) conjugated to horseradish peroxidase (HRP) diluted 5000X to 10000X in PBST, for 1 to 2 hours at room temperature. All the secondary antibodies used were from Santa Cruz Biotechnology. The blots were then washed again in PBST thrice and incubated with Western Lightning Plus ECL reagent (Perkin Elmer) for 1min at room temperature. The membrane was exposed at Kodak Biomax MS film (Kodak) for 30 sec to 15 min depending on the intensity of the signal, and the films were developed in the Kodak X-ray Processor.

3.3.5 Antibodies

Table 3.1 List of antibodies used in western blot.

Antibody	Company	Source	Dilution
HDAC1	Upstate	Mouse monoclonal	1:1,000
HDAC2	Upstate	Mouse monoclonal	1:1,000
GAPDH	Santa Cruz	Mouse monoclonal	1:5,000
Actin	Sigma	Mouse monoclonal	1:10,000
Caspase 3	Cell Signaling	Mouse monoclonal	1:1,000
PARP	Cell Signaling	Rabbit polyclonal	1:1,000
P53	Santa Cruz	Mouse monoclonal	1:2,000
P21	Santa Cruz	Mouse monoclonal	1:500
NMES	Santa Cruz	Rabbit Polyclonal	1:500
CYGB	Santa Cruz	Rabbit Polyclonal	1:500
MAGEC2	Santa Cruz	Goat Polyclonal	1:500
PLCgamma2	Santa Cruz	Rabbit Polyclonal	1:500
LOX	Abcam	Rabbit Polyclonal	1:1000
LOXL4	Abcam	Rabbit Polyclonal	1:1000
GalR2	Abcam	Rabbit Polyclonal	1:500

3.3.6 Densitometry

To quantify the Western Blot results, the GS800 Calibrated Densitometer (BioRad) was used to scan the films after developing. A software (QuantityOne) was used to quantify the intensity of the bands on the films.

3.4 Design of siRNA to knockdown HDAC1 and 2

Both HDAC1 and HDAC2 belong to class I of mammalian HDAC and are highly homologous. Their mRNA coding sequence shared 74.3% identity upon alignment (Figure 3.1). Stealth RNAi siRNA was purchased from Invitrogen (Life Technologies, USA). Three siRNA sequences (A, B and C) each targeting different regions along the mRNAs of HDAC1 and HDAC2 were shown in Figure 3.2 and Figure 3.3 respectively. A universal non-silencing control (Scr) that has sequences of matching GC content was also purchased from Invitrogen as recommended by the manufacturer's protocol.

```
EMBOSS_001         1 atggcg--cagacgcag-ggcacccggaggaaagtctgttactactacga     47
                     ||||||  |||.|...| |||..|...|..||||||||.||||||||||
EMBOSS_001         1 atggcgtacagtcaaggaggcggcaaaaaaaaagtctgctactactacga     50
EMBOSS_001        48 cggggatgttggaaattactattatggacaaggccacccaatgaagcctc     97
                     |||.|||.||||||||||.||||||||||.||.||.||.||||||||||
EMBOSS_001        51 cggtgatattggaaattattattatggacagggtcatcccatgaagcctc    100
EMBOSS_001        98 accgaatccgcatgactcataatttgctgctcaactatggtctctaccga    147
                     |..|||||||||||||.|||||.||||||.|.||.||||||..|.|||.||
EMBOSS_001       101 atagaatccgcatgacccataacttgctgttaaattatggcttatacaga    150
EMBOSS_001       148 aaaatggaaatctatcgccctcacaaagccaatgctgaggagatgaccaa    197
                     ||||||||||.|||.|.||.||.|||||||.|||.||.||.||||||.||
EMBOSS_001       151 aaaatggaaatatataggccccataaagccactgccgaagaaatgacaaa    200
EMBOSS_001       198 gtaccacagcgatgactacattaaattcttgcgctccatccgtccagata    247
                     .||.||||||.||||||.||.||.||||||..|.||.||.||..|.|||||||
EMBOSS_001       201 atatcacagtgatgagtatatcaaatttctacggtcaataagaccagata    250
EMBOSS_001       248 acatgtcggagtacagcaagcagatgcagagattcaacgttggtgaggac    297
                     |||||||.||||||.||.||||||||||||.|.|||.||.||||||.||.||.
EMBOSS_001       251 acatgtctgagtatagtaagcagatgcatatatttaatgttggagaagat    300
EMBOSS_001       298 tgtccagtattcgatggcctgtttgagttctgtcagttgtctactggtgg    347
                     |||||||..||.||||||.||.||||||||.||||||.|.||.||||||.||
EMBOSS_001       301 tgtccagcgtttgatggactctttgagttttgtcagctctcaactggcgg    350
EMBOSS_001       348 ttctgtggcaagtgctgtgaaacttaataagcagcagacggacatcgctg    397
                     |||.||.||..|.||||||||..|.||....||.||||||.||.||.||||
EMBOSS_001       351 ttcagttgctggagctgtgaagttaaaccgacaacagactgatatggctg    400
EMBOSS_001       398 tgaattgggctgggggcctgcaccatgcaaagaagtccgaggcatctggc    447
                     |.||||||||||||.||..|.||.||||||.||||||.|.|||.||||||.||.
EMBOSS_001       401 ttaattgggctggaggattacatcatgctaagaaatacgaagcatcagga    450
EMBOSS_001       448 ttctgttacgtcaatgatatcgtcttggccatcctggaactgctaaagta    497
                     ||||||||||||.||||||||.||..|.||||||||.|||.|.||||||||
EMBOSS_001       451 ttctgttacgttaatgatattgtgcttgccatccttgaattactaaagta    500
EMBOSS_001       498 tcaccagagggtgctgtacattgacattgatattcaccatggtgacggcg    547
                     |||.||||||.||..|.||.||||||.||.||||||||.||||||||.||.|
EMBOSS_001       501 tcatcagagagtcttatatattgatatagatattcatcatggtgatggtg    550
EMBOSS_001       548 tggaagaggccttctacaccacggaccgggtcatgactgtgtcctttcat    597
                     |.||||||.||.||.||.||.||.||.||.||.||||||.||.||.||.|||
EMBOSS_001       551 ttgaagaagctttttatacaacagatcgtgtaatgacggtatcattccat    600
EMBOSS_001       598 aagtatggagagtacttcccaggaactggggacctacgggatatcggggc    647
                     ||.||||||.||.||||||.||.||.||.||.||.|||.|..||||||||.||.||
EMBOSS_001       601 aaatatggggaatactttcctggcacaggagacttgagggatattggtgc    650
EMBOSS_001       648 tggcaaaggcaagtattatgctgttaactacccgctccgagacgggattg    697
                     |||.|||||||||.||.||||||||.||.|..||..|..|.||.||.||.|
EMBOSS_001       651 tggaaaaggcaaatactatgctgtcaattttccaatgtgtgatggtatag    700
EMBOSS_001       698 atgacgagtcctatgaggcc-attttcaagccggtcatgtccaaagtaat    746
                     ||||.||||||.|||| |||. ||.||.||||||..|.||.||.||.||.||
EMBOSS_001       701 atgatgagtcatatg-ggcagatatttaagcctattatctcaaaggtgat    749
EMBOSS_001       747 ggagatgttccagcctagtgcggtggtcttacagtgtggctcagactccc    796
                     ||||||||..||.||||||||.||||||.||||||||||..|||||||..
EMBOSS_001       750 ggagatgtatcaacctagtgctgtggtattacagtgtggtgcagactcat    799
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EMBOSS_001       797 tatctggggatcggttaggttgcttcaatctaactatcaaaggacacgcc    846
                     |||||||.|||.|..|.|||||.||||||||||..|||||||.||.||.
EMBOSS_001       800 tatctggtgatagactgggttgtttcaatctaacagtcaaaggtcatgct    849
EMBOSS_001       847 aagtgtgtggaatttgtcaagagctttaacctgcctatgctgatgctggg    896
                     ||.||||||.|||.||||.||.|..||||||.|.||..|.|||||||.||
EMBOSS_001       850 aaatgtgtagaagttgtaaaaacttttaacttaccattactgatgcttgg    899
EMBOSS_001       897 aggcggtggttacaccattcgtaacgttgcccggtgctggacatatgaga    946
                     |||.||||||.||||||.||.||||||.||||||.||.||.||||||||||||||
EMBOSS_001       900 aggaggtggctacacaatccgtaatgttgctcgatgttggacatatgaga    949
EMBOSS_001       947 cagctgtggccctggatacggagatccctaatgagcttccatacaatgac    996
                     |.||.||.||||||.|||...||||||.||.||||||.|.||||||.||||||.
EMBOSS_001       950 ctgcagttgcccttgattgtgagattcccaatgagttgccatataatgat    999
EMBOSS_001       997 tactttgaatactttggaccagatttcaagctccacatcagtccttccaa   1046
                     ||||||||.||.||||||||||||.||||||.||.||.||.||||||||.||
EMBOSS_001      1000 tactttgagtattttggaccagacttcaaactgcatattagtccttcaaa   1049
EMBOSS_001      1047 tatgactaaccagaacacgaatgagtacctggagaagatcaaacagcgac   1096
                     .||||||.||||||||||||....||.||..||||.||||||.||||||||..
EMBOSS_001      1050 catgacaaaccagaacactccagaatatatggaaaagataaaacagcgtt   1099
EMBOSS_001      1097 tgtttgagaaccttagaatgctgccgcacgcacctggggtccaaatgcag   1146
                     |||||||.||..|..|.|||.|.||.||.||||||||.||||||.||||||.
EMBOSS_001      1100 tgtttgaaaatttgcgcatgttacctcatgcacctggtgtccagatgcaa   1149
EMBOSS_001      1147 gcgattcctgaggacgccatccctgaggagagtggcgatgaggacgaaga   1196
                     ||.||||||.||.||.||..|.|.|||.||.||||||.||||||.||.|.|||
EMBOSS_001      1150 gctattccagaagatgctgttcatgaagacagtggagatgaagatggaga   1199
EMBOSS_001      1197 cgaccctgacaagcgcatctcgatctgctcctctgacaaacgaattgcct   1246
                     .||.||.||||||.|.||.||.||..|..|.||.||||||.||.||.||.|
EMBOSS_001      1200 agatccagacaagagaatttctattcgagcatcagacaagcggatagctt   1249
EMBOSS_001      1247 gtgaggaagagttctccgattctgaagaggagggagagggggccgcaag   1296
                     ||||.||||||.||||||.||||||||.||.||.||||||.||.||.|| |||
EMBOSS_001      1250 gtgatgaagaattctcagattctgaggatgaaggagaaggaggtcg-aag   1298
EMBOSS_001      1297 aactcttcc--aacttcaaaaa--agccaagagagtcaaaacagaggatg   1342
                     ||.|.|..|  |.|.|.|.|||  |||.||||.||..|.||..||.||.|
EMBOSS_001      1299 aaatgtggctgatcataagaaaggagcaaagaaagctagaattgaagaag   1348
EMBOSS_001      1343 aaaaagagaaagacccagaggagaagaa---agaagtcaccgaagaggag   1389
                     |.|   |||||||..|||||||.||.||   |||.||.|..|||||.||.
EMBOSS_001      1349 ata---agaaagaaacagaggacaaaaaaacagacgttaaggaagaagat   1395
EMBOSS_001      1390 aaaaccaagga-------ggag--aagccagaagccaaaggggtcaa---   1427
                     |||.|||||||       ||.|  ||..||||..|||||||...|||
EMBOSS_001      1396 aaatccaaggacaacagtggtgaaaaaacagataccaaaggaaccaaatc   1445
EMBOSS_001      1428 ggaggaggtcaagttggcctga   1449
                     .||..||.|||......|||||
EMBOSS_001      1446 agaacagctcagcaacccctga   1467
```

Figure 3.1 Alignment of coding sequence of HDAC1 (top) and HDAC2 (bottom)

siRNA 1A
siRNA 1B
siRNA 1C

1 gagcggagcc gcgggcggga gggcggacgg accgactgac ggtagggacg ggaggcgagc
61 aag***atg***gcgc agacgcaggg cacccggagg aaagtctgtt actactacga cggggatgtt
121 ggaaattact attatggaca aggccaccca atgaagcctc accgaatccg catgactcat
181 aatttgctgc tcaactatgg tctctaccga aaaatggaaa tctatcgccc tcacaaagcc
241 aatgctgagg agatgaccaa gtaccacagc gatgactaca ttaaattctt gcgctccatc
301 cgtccagata acatgtcgga gtacagcaag cagatgcaga gattcaacgt tggtgaggac
361 tgtccagtat tcgatggcct gtttgagttc tgtcagttgt ctactggtgg ttctgtggca
421 agtgctgtga aacttaataa gcagcagacg gacatcgctg tgaattgggc tgggggcctg
481 caccatgcaa agaagtccga ggcatctggc ttctgttacg tcaatgatat cgtcttggcc
541 atcctggaac tgctaaagta tcaccagagg gtgctgtaca ttgacattga tattcaccat
601 ggtgacggcg tggaagaggc cttctacacc acggaccggg tcatgactgt gtcctttcat
661 aagtatggag agtacttccc aggaactggg gacctacggg atatcggggc tggcaaaggc
721 aagtattatg ctgttaacta cccgctccga gacgggattg atgacgagtc ctatgaggcc
781 attttcaagc cggtcatgtc caaagtaatg gagatgttcc agcctagtgc ggtggtctta
841 cagtgtggct cagactccct atctggggat cggttaggtt gcttcaatct aactatcaaa
901 ggacacgcca agtgtgtgga atttgtcaag agctttaacc tgcctatgct gatgctggga
961 ggcggtggtt acaccattcg taacgttgcc cggtgctgga catatgagac agctgtggcc
1021 ctggatacgg agatccctaa tgagcttcca tacaatgact actttgaata ctttggacca
1081 gatttcaagc tccacatcag tccttccaat atgactaacc agaacacgaa tgagtacctg
1141 gagaagatca aacagcgact gtttgagaac cttagaatgc tgccgcacgc acctggggtc
1201 caaatgcagg cgattcctga ggacgccatc cctgaggaga gtggcgatga ggacgaagac
1261 gaccctgaca agcgcatctc gatctgctcc tctgacaaac gaattgcctg tgaggaagag
1321 ttctccgatt ctgaagagga gggagagggg ggccgcaaga actcttccaa cttcaaaaaa
1381 gccaagagag tcaaaacaga ggatgaaaaa gagaaagacc cagaggagaa gaaagaagtc
1441 accgaagagg agaaaaccaa ggaggagaag ccagaagcca aaggggtcaa ggaggaggtc
1501 aagttggcc***t ga***atggacct ctccagctct ggcttcctgc tgagtccctc acgtttcttc
1561 cccaacccct cagattttat attttctatt tctctgtgta tttatataaa aatttattaa
1621 atataaatat ccccagggac agaaaccaag gccccgagct cagggcagct gtgctgggtg
1681 agctcttcca ggagccacct tgccacccat tcttcccgtt cttaactttg aaccataaag
1741 ggtgccaggt ctgggtgaaa gggatacttt tatgcaacca taagacaaac tcctgaaatg
1801 ccaagtgcct gcttagtagc tttggaaagg tgcccttatt gaacattcta gaaggggtgg
1861 ctgggtcttc aaggatctcc tgtttttttc aggctcctaa agtaacatca gccatttta
1921 gattggttct gttttcgtac cttcccactg gcctcaagtg agccaagaaa cactgcctgc
1981 cctctgtctg tcttctccta attctgcagg tggaggttgc tagtctagtt tccttttga
2041 gatactattt tcatttttgt gagcctcttt gtaataaaat ggtacatttc t

Figure 3.2 HDAC1 mRNA and location of siRNA sequences. Three siRNA sequences against HDAC1 (1A, 1B, and 1C) were purchased from Invitrogen and their locations along the HDAC1 mRNA sequence were indicated.

siRNA 2A
siRNA 2B
siRNA 2C

1 cgccgagctt tcggcacctc tgccgggtgg taccgagcct tcccggcgcc ccctcctctc
61 ctcccaccgg cctgcccttc cccgcgggac tatcgccccc acgtttccct cagccctttt
121 ctctcccggc cgagccgcgg cggcagcagc agcagcagca gcagcaggag gaggagcccg
181 gtggcggcgg tggccgggga gccc***atg***gcg tacagtcaag gaggcggcaa aaaaaaagtc
241 tgctactact acgacggtga tattggaaat tattattatg gacagggtca tcccatgaag
301 cctcatagaa tccgcatgac ccataacttg ctgttaaatt atggcttata cagaaaaatg
361 gaaatatata ggccccataa agccactgcc gaagaaatga caaaatatca cagtgatgag
421 tatatcaaat ttctacggtc aataagacca gataacatgt ctgagtatag taagcagatg
481 catatattta atgttggaga agattgtcca gcgtttgatg gactctttga gttttgtcag
541 ctctcaactg gcggttcagt tgctggagct gtgaagttaa accgacaaca gactgatatg
601 gctgttaatt gggctggagg attacatcat gctaagaaat acgaagcatc aggattctgt
661 tacgttaatg atattgtgct tgccatcctt gaattactaa agtatcatca gagagtctta
721 tatattgata tagatattca tcatggtgat ggtgttgaag aagcttttta tacaacagat
781 cgtgtaatga cggtatcatt ccataaatat ggggaatact ttcctggcac aggagacttg
841 agggatattg gtgctggaaa aggcaaatac tatgctgtca attttccaat gtgtgatggt
901 atagatgatg agtcatatgg gcagatattt aagcctatta tctcaaaggt gatggagatg
961 tatcaaccta gtgctgtggt attacagtgt ggtgcagact cattatctgg tgatagactg
1021 ggttgtttca atctaacagt caaaggtcat gctaaatgtg tagaagttgt aaaaactttt
1081 aacttaccat tactgatgct tggaggaggt ggctacacaa tccgtaatgt tgctcgatgt
1141 tggacatatg agactgcagt tgcccttgat tgtgagattc ccaatgagtt gccatataat
1201 gattactttg agtattttgg accagacttc aaactgcata ttagtccttc aaacatgaca
1261 aaccagaaca ctccagaata tatggaaaag ataaaacagc gtttgtttga aaatttgcgc
1321 atgttacctc atgcacctgg tgtccagatg caagctattc cagaagatgc tgttcatgaa
1381 gacagtggag atgaagatgg agaagatcca gacaagagaa tttctattcg agcatcagac
1441 aagcggatag cttgtgatga agaattctca gattctgagg atgaaggaga aggaggtcga
1501 agaaatgtgg ctgatcataa gaaaggagca aagaaagcta gaattgaaga agataagaaa
1561 gaaacagagg acaaaaaaac agacgttaag gaagaagata aatccaagga caacagtggt
1621 gaaaaaacag ataccaaagg aaccaaatca gaacagctca gcaacccc***tg a***atttgacag
1681 tctcaccaat ttcagaaaat cattaaaaag aaaatattga aaggaaaatg ttttcttttt
1741 gaagacttct ggcttcattt tatactactt tggcatggac tgtatttatt ttcaaatggg
1801 actttttcgt ttttgttttt ctgggcaagt tttattgtga gattttctaa ttatgaagca
1861 aaatttcttt tctccaccat gctttatgtg atagtattta aaattgatgt gagttattat
1921 gtcaaaaaaa ctgatctatt aaagaagtaa ttggcctttc tgagctgaaa aaaaaaaaaa
1981 aaaag

Figure 3.3 HDAC2 mRNA and location of siRNA sequences. Three siRNA sequences against HDAC2 (2A, 2B, and 2C) were purchased from Invitrogen and their locations along the HDAC2 mRNA sequence were indicated.

3.5 Colony Formation Assay

Between 24 to 48 hours after transfection or drug treatment, cells were harvested by trypsinization and counted using the hemocytometer. Depending on the cell type, 1000 to 5000 cells were plated into each well in a 6 well plate, in triplicate wells. The plates were returned to the incubator.

At the end of 7 to 14 days, depending on the cell type, the wells were washed in 1X PBS and the colonies stained with crystal violet solution. The plates were then scanned and the images were analysed using the ImageJ (NIH) software to measure the number of colonies formed in each well.

3.6 WST-1 Cell Proliferation Assay

To measure cell proliferation, the colorimetric assay was performed using WST-1 reagent (Roche).

3.6.1 Cell plating

Twelve hours post-transfection, cells were trypsinized, counted by hemocytometer, and replated at 500 cells/well in a 96well plate. This was done for 6 plates with 5 replicate wells for each sample on each plate. These plates were placed in the humidified 37°C incubator with 5% CO_2 until they are ready to be assayed.

3.6.2 WST-1 Assay

Thirty-six hours post-transfection (24h after replating), 10µl of WST reagent was added to each well containing 100µl of media. The plate was returned to humidified 37°C incubator with 5% CO_2 for 4 hours before the absorbance was read at 460nm using a spectrophotometer. A “blank” well with only the media and WST

reagent but without cells was used as negative control. Subsequently, at 24 hours interval, one plate will be assayed until all the 6 plates were read after 6 days.

3.7 Cell cycle analysis

3.7.1 Collection of cells for fixation

Cells were washed in 1X PBS, trpysinized, and collected. Both the live adherent and dead floating cells were collected and pelleted at 2500 RPM for 5 min at 4°C. They were washed 1X PBS, fixed in cold 70% ethanol and stored in -20°C for overnight.

Staining with propidium iodide:

The fixed cells were centrifuged at 2500 RPM for 5 min at 4°C and washed once in 1X PBS. They were then resuspended in 500µl propidium iodide (PI) staining solution (0.1% triton-X, 0.2mg/ml RNaseA, 0.02mg/ml propidium iodide, in 1XPBS) and incubated for 15 min at 37°C in the dark.

3.7.2 Flow cytometry

The PI-stained cells were filtered through a 40µm filter before being run through a Beckman Coulter Epics Altra at the Flow Cytometry Unit at National University Medical Institute. Ten thousand cells were analysed for each sample to generate a cell cycle profile. Analysis was done using the WinMDI software.

3.8 Cloning of pcDNA-HDAC1 and pcDNA-HDAC2 plasmids

3.8.1 PCR to amplify DNA and DNA fragment purification by gel extraction

The full length HDAC1 and HDAC2 coding sequences were amplified from the cDNA of HCT116 cells using primers engineered with the BamH1 and Xho1 restriction sites at the 5' and 3' ends respectively. The PCR products were run on a

1.2% agarose gel in 0.5X TBE, at 100V for 1hour. The DNA fragments were excised under brief UV light and gel extraction was done using the QIAquick Gel Extraction kit (Qiagen) according to the manufacturer's instructions. The purified PCR products of HDAC1 and HDAC2, as well as the pcDNA3.1 vector (Invitrogen), were cut using BamH1 and Xho1 enzymes from Promega for 3 hours at 37°C before the enzymes were inactivated at 65°C for 15 min. The digested fragments of HDAC1, HDAC2 and pcDNA3.1 vector were run on a 1.2% agarose gel and gel extraction was done using a kit (Qiagen) according to the manufacturer's instructions.

3.8.2 Ligation

The purified HDAC1 and HDAC2 fragments were ligated into the pcDNA3.1 vector using T4 Ligase (Promega) and the 2X Rapid Ligase Buffer (Promega) in 4°C for 16 hours.

3.8.3 Transformation

Ten percent of each ligation mixture was incubated with 50µl of freshly thawed XL1Blue competent cells on ice for 30 min, heat-shocked at 42°C for 45 sec and immediately cooled in ice for 2 min. The transformed cells were then cultured in 250µl of SOC medium in an orbital shaker at 37°C for 1 hour. One hundred µl of the bacteria culture were then plated onto LB agar plates containing ampicillin (100µg/ml), IPTG (100nM) and X-gal (1.75mg) and grown for 16 hours in 37°C incubator. The next day, white colonies were picked from each plate and cultured in 3ml LB media with ampicillin (100µg/ml) in an orbital shaker at 37°C for 16 hours.

3.8.4 Plasmid miniprep

Bacterial pellets were obtained for each sample by centrifuging 2ml of the culture in a tube. The remaining 1ml culture was stored in 4°C for future inoculation into larger culture volume if the sample was verified to be positive.

Plasmid DNA was extracted from the bacterial culture using the QIAprep Miniprep kit (Qiagen). Briefly, the bacterial pellets were resuspended in a pre-lysis buffer and a lysis solution was then added until a clear and viscous lysate was obtained. With the addition of a neutralizing buffer, the bacterial membrane, proteins and chromosomal DNA would be precipitated and spun down at 14,000x g for 10 min. The supernatant, which contained the plasmid DNA, was allowed to bind to the spin column provided in the kit and washed. This plasmid DNA was eluted with 30μl of buffer.

3.8.5 Verification of positive clones

The plasmid DNA from each sample was digested with BamH1 and Xho1 (Promega) for 3 hours at 37°C. The digested reaction was run on 1.2% agarose gel and the sizes of the bands were verified to match that of the DNA insert and pcDNA vector.

3.8.6 Plasmid midiprep

The 1ml bacteria cultures from the positive clones were inoculated into 50ml of LB media containing ampicillin (100μg/ml) and incubated in an orbital shaker at 37°C for 16 hours. Plasmid DNA was extracted from the bacteria culture using the HiSpeed Plasmid Midi Purification kit (Qiagen). The 50ml bacteria culture was centrifuged and the pellet was resuspended in buffer P1 (with added RNase A), followed by the addition of buffer P2 for lysis and P3 for neutralization and precipitation of proteins and chromosomal DNA. The precipitates were filtered and the lysate were allowed to bind to the HiSpeed Tip, washed, and eluted with buffer QF. The plasmid DNA was then precipitated using isopropanol and allowed to bind to the QIA precipitator. Elution was done with TE buffer and the concentration of the DNA was measured using UV spectrophotometry.

3.8.7 Sequencing reaction

The plasmids were sequenced to ensure that there is no mutation in the DNA. The Big-Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) was used to perform the sequencing reaction, which was read using the automated ABI Prism 377 sequencer (Amersham) at the National University of Singapore Medical Institutes' sequencing facility.

The sequencing reaction consisted of 300ng of DNA, 6μl of Terminator Ready Reaction Mix containing DNA polymerase, buffer, and dideoxynucleotide (ddATP, ddVTP, ddGTP and ddTTP), 2μl of 5X sequencing buffer, and 3.2pmol of sequencing primer. The final volume was made up to 20μl using deionized water. The mixture was subjected to 25 cycles of thermal sequencing on a GeneAmp 2720 system (PE Applied Biosystems): 95°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. The extension products were purified by ethanol precipitation and the pellet was air-dried before being reconstituted in sequencing loading buffer and sequenced using the ABI Prism 377 sequencer.

3.9 Site-directed mutagenesis

To generate enzyme-dead mutants of HDAC1 and HDAC2 plasmids, QuikChangeII site-directed mutagenesis kit (Stratagene) was used to mutate 2 nucleotides within the catalytic domain of the enzyme based on Hassig et al. (Hassig et al., 1998). This mutation changed the amino acid number 141 from a histidine to an alanine in the HDAC1 protein, which was verified by Hassig et al. to maintain native folding but confer no HDAC enzyme activity. Because this catalytic domain is identical between HDAC1 and HDAC2, we also mutated the same 2 nucleotides in order to change amino acid number 142 from histidine to alanine to generate a

HDAC2 enzyme dead mutant. Primers were designed using the program provided on the Stratagene website http://www.stratagene.com/sdmdesigner/default.aspx

Table 3.2 Primer sequences used for generating HDAC1 and HDAC2 mutants.

Mutant	Primer name	Primer sequence (5'to 3')
HDAC1	H141A	ctgggggcctgcacgctgcaaagaagtccg
	H141A_antisense	cggacttctttgcagcgtgcaggcccccag
HDAC2	H142A	gggctggaggattacatgctgctaagaaatacgaagc
	H142A_antisense	gcttcgtatttcttagcagcatgtaatcctccagccc

These primers were synthesized (Sigma-Proligo) and HPLC purified. The mutant strand synthesis reaction was set up as below:

5µl of 10X reaction buffer

Xµl (50ng) of dsDNA template (either pcDNA-HDAC1 or pcDNA-HDAC2)

Xµl (125ng) of primer#1 (H141A or H142A)

Xµl (125ng) of primer#2 (H141A_antisense or H142A_antisense)

1µl of dNTP mix

ddwater to a final volume of 50µl

then add 1µl PfuUltra HF DNA polymerase (2.5U/µl)

Table 3.3 Cycling parameters used for site-directed mutagenesis.

Segment	Cycles	Temperature	Time
1	1	95°C	30 sec
2	16	95°C	30 sec
		55°C	1 min
		68°C	5 min

At the end of the temperature cycling, the reactions were placed on ice for 2 min. Then 1µl of the Dpn1 restriction enzyme was added to each reaction and incubated at 37°C for 1 hour to digest the parental nonmutated supercoiled dsDNA. Subsequently, the reactions were used in transformation of the XL1-Blue supercompetent cells

provided in the kit and positive clones were picked for plasmid extraction using Miniprep and Midiprep as described previously. Verification was performed by sequencing of the plasmids to ensure that the mutations were achieved.

3.10 Immunoprecipitation

3.10.1 Cell lysis

Cells were washed twice in cold 1X PBS before being lysed by cold RIPA Buffer (1% NP40, 1% Sodium Deoxycholate, 0.1% SDS, 0.15M sodium chloride, 50mM Tris pH8) with freshly added cocktail protease inhibitor (Roche). After incubating for 15 min on a rotator at 4°C, the lysed cells were scraped and transferred to a tube for further rotation at 4°C for 10 min. The samples were centrifuged at 12,000 RPM at 4°C for 15 min and the supernatant was collected as the cell lysate. Quantification of protein was performed as described in the previous section. Equal amount of protein (1-5mg) was taken from each lysate sample and made up to the same volume for immunoprecipitation.

3.10.2 Binding with antibodies and beads

One μg of primary antibody was added to each sample. As a negative control, either mouse IgG or rabbit IgG was used depending on the source of the antibody used. The samples were incubated for 2 hours on a rotator at 4°C. Protein G beads (Amersham) was washed and equilibrated with RIPA buffer and added to each sample, to be incubated for 2 hours on a rotator at 4°C.

3.10.3 Elution

The beads were washed in cold 1X PBS for 5 min for 3 times. Loading buffer was diluted to be 2X and 35μl was added to each sample, which was placed in a heat block at 95°C for 4 min to elute the immunoprecipitates. The immunoprecipitates

were carefully loaded into the wells of a polyacrylamide gel. The subsequent steps were described in the previous section on Western Blot.

3.11 HDAC Activity Assay

3.11.1 Extraction of nuclear protein

Cells were washed in 1X PBS and collected by trypsinization and pelleted at 1000 RPM for 5 min at 4°C. The cell pellet was lysed in a cold lysis buffer (0.65M sucrose, 20mM Tris pH8.0, 10mM magnesium chloride, 2% Triton-X) on ice for 15 min. The samples were centrifuged at 2000 RPM for 5 min at 4°C and the supernatant containing the cytoplasmic protein was removed. The nuclear pellet was resuspended and lysed in ice for 20 min in a NT Buffer (50mM Tris pH7.4, 100mM sodium chloride, 5mM magnesium chloride, 5mM calcium chloride, 1% NP40, 1% Triton-X, 10units of DNaseI) with freshly added cocktail protease inhibitor (Roche). The samples were centrifuged at 13,000 RPM for 15 min at 4°C and the supernatant from each was collected as the nuclear lysate. Protein quantification was done as described in the previous section.

3.11.2 Fluorometric HDAC Activity Assay

The global histone deacetylase activity of each sample was measured using the HDAC Assay Kit according to manufacturer's instructions (Upstate). Briefly, 5μg of the nuclear protein was incubated with the substrate in a half-volume plate provided in the kit at 37°C for 30 min. An activator solution with the HDAC inhibitor Trichostatin (TSA) was added to stop the enzymatic reaction and to give a fluorometric signal, to be read on a fluorescence plate reader with an excitation of 360nm and emission of 460nm. Each sample was done in duplicates and the average counts per second (CPS) was taken.

To measure the HDAC activity of HDAC1 and HDAC2 in a cell lysate sample specifically, immunoprecipition was performed using the HDAC1 and HDAC2 antibodies respectively, as described in the previous section. Instead of eluting the immunoprecipitates from the beads using the loading buffer, the beads were washed in the HDAC Assay Buffer provided in the kit and incubated with the substrate at 37°C for 30 min. The samples were centrifuged at 8000x g for 30 sec to pellet the beads and the supernatant was transferred to the plate to be incubated with the activator solution and read using the fluorescence plate reader.

3.12 RNA isolation

Total RNA was extracted from cells using the Qiagen RNeasy Mini Kit according to the manufacturer's instructions (Qiagen). Briefly, cells were harvested by trypsinization and washed in 1X PBS. They were then lysed by 350µl RLT buffer containing 10µl beta-mercaptoethanol and homogenized using needle and syringe. Equal volume of 70% ethanol was added and each sample was applied to the RNeasy minispin column for centrifugation at 10,000x g for 30 sec. This was followed by 2 washing steps with 500µl of RPE buffer and a drying step to remove all traces of the ethanol in the buffer by centrifugation at 10,000x g for 2 min. Finally the RNA was eluted with 35µl of RNase-free water. Quantification of RNA was done at 260nm.

3.13 Microarray

HEB3B cells were transfected with siRNA against HDAC1 or/and HDAC2 as described in the previous section. Seventy-two hours after transfection, cells were harvested for protein and RNA extraction. After verifying by Western blot that the efficiency of knockdown was satisfactory, the RNA was quantified. In addition,

another set of HEP3B cells were treated with either 2000nM PXD101 (HDAC inhibitor) or vehicle control for 24hours and RNA extraction was done. Gene expression profiles of samples after each treatment were examined using the Illumina HumanRef-8 v3 beadchip which has 24,526 probes in each array. Analysis was done using Genespring version 2. Gene ontogeny pie chart was generated based on the Gene Ontogeny (GO) classification by molecular function, biological process and cellular component using the Protein ANalysis THrough Evolutionary Relationships (PANTHER) classification system (Thomas et al., 2003).

3.14 Real-time RT-PCR

3.14.1 cDNA synthesis

One μg of RNA was used for cDNA synthesis. The reaction was set up as follows: 5μl of RNA, 1.25μl of Oligo dT, 1.25μl dNTP, 7.5μl DEPC water. This was incubated for 5min at 65^{o}C before adding the following: 5μl first strand buffer (Invitrogen), 2.5μl 0.1M DTT, 1.25μl DEPC water, 1.25μl ImpromII Reverse Transcriptase (Promega). The reaction was incubated at 42^{o}C for 60 min. For subsequent PCR, 1 out of 25μl total volume was used.

3.14.2 Quantitative PCR

Using the Roche LightCycler SYBR Green DNA amplification kit (Roche Diagnostics, Mannheim, Germany), quantitative PCR was performed according to manufacturer's specification on the Roche LightCycler 480 machine. Each reaction sample was set up in duplicates in a special 96 well plate (Roche) with 10μl of the 2X reaction mix, 1μl of cDNA, 1μl of forward primer (10μM) and 1μM of reverse primer (10μM) and 7μl of water.

Table 3.4 List of primers used in RT-PCR.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
MAGEC2	AGGCGCGAATCAAGTTAG	CTCCTCTGCTGTGCTGAC
NMES1	AGGAACTCATTCCCTTGGTG	TCCACAGTTTCCCAAGGTTC
NOTCH3	AAGGACGTGGCCTCTGGT	TCAGGCTCTCACCCTTGG
CYGB	TGGCCATCCTGGTGAGGTT	CAGGCGTGCTTCCGCAGCT
DYRK4	GCTGCATCACGGCGGAGTTGTA	TCCGGGGTCATGCGAAGAGAAG
GLUT3	GCATATGATAGGCCTTGGAG	CATTGGTGGTGGTCTCCTTA
LOX	GAACGCTTAAGTCATCATTACTTG	TGGCTCATTCATTTAGATAATACTGA
LOXL4	AGGGCCTCTGCCAAGGAAATAA	ATCAAGCAGGAATGGTGGCCTT
GALR2	TTCCTCATCTTCCTCACCATG	ATGGCACACGGTCAGGTT
GAPDH	AGCAATGCCTCCTGCACCACCAAC	CCGGAGGGGCCATCCACAGTCT
HDAC1	AACTGGGGACCTACGG	ACTTGGCGTGTCCTT
HDAC2	GTTGCTCGATGTTGGAC	CCAGGTGCATGAGGTA

The amplification involved an initial denaturation step of 2 min, followed by 40 cycles of denaturation step at 95°C for 0 sec, annealing at 60°C for 10 sec and extension at 72°C for 10 sec. At the end of each cycle, the SYBR Green fluorescence emitted was measured. The crossing point (CP) for each reaction was determined by the software based on the fluorescence signal. The mRNA expression for each sample was calculated according to the Roche Applied Science Technical Note No. LC 13/2001, and normalized against the internal control GAPDH.

CHAPTER 4

RESULTS

CHAPTER 4 RESULTS

4.1 HDAC1 and HDAC2 expression in human liver cancer

4.1.1 HDAC1 and 2 expression was increased in human hepatocellular carcinoma protein extracts

Protein extracts from 28 pairs of hepatocellular carcinoma and their matched adjacent non-tumor tissue were obtained from the National University Hospital Tissue Repository with Institutional Review Board (IRB) approval. The protein expression of HDAC1 and HDAC2 were examined by Western blot as shown in Figure 4.1, with GAPDH as the loading control. The bands of HDAC1 and HDAC2 on the blots were then quantified by densitometry and normalized against that of GAPDH. Figure 4.2 shows the fold increase in HDAC1 and HDAC2 protein expression in the tumor versus the matched non-tumor tissues. Of the 28 pairs of samples, 8 were not quantified because the band on the blot was not well-defined enough to be imaged accurately for densitometry. Altogether, 68.4% (13 out of 19 pairs) showed an upregulation of HDAC1 in the tumor compared to the matched non-tumor tissue, while 85.2% (23 out of 27) showed upregulation of HDAC2 in the tumor compared to the matched non-tumor tissue, and 63.2% (12 out of 19 pairs) showed upregulation of both HDAC1 and 2. Therefore, majority of the matched hepatocellular carcinoma tissues have upregulated protein expression HDAC1 and 2.

4.1.2 HDAC1 and 2 expression was increased in human hepatocellular carcinoma tissues by tissue microarray analysis

Tissue microarray from 179 pairs of hepatocellular carcinoma and their matched adjacent non-tumor tissue were obtained from the Department of Pathology at the National University Hospital with IRB approval. Each sample was from a

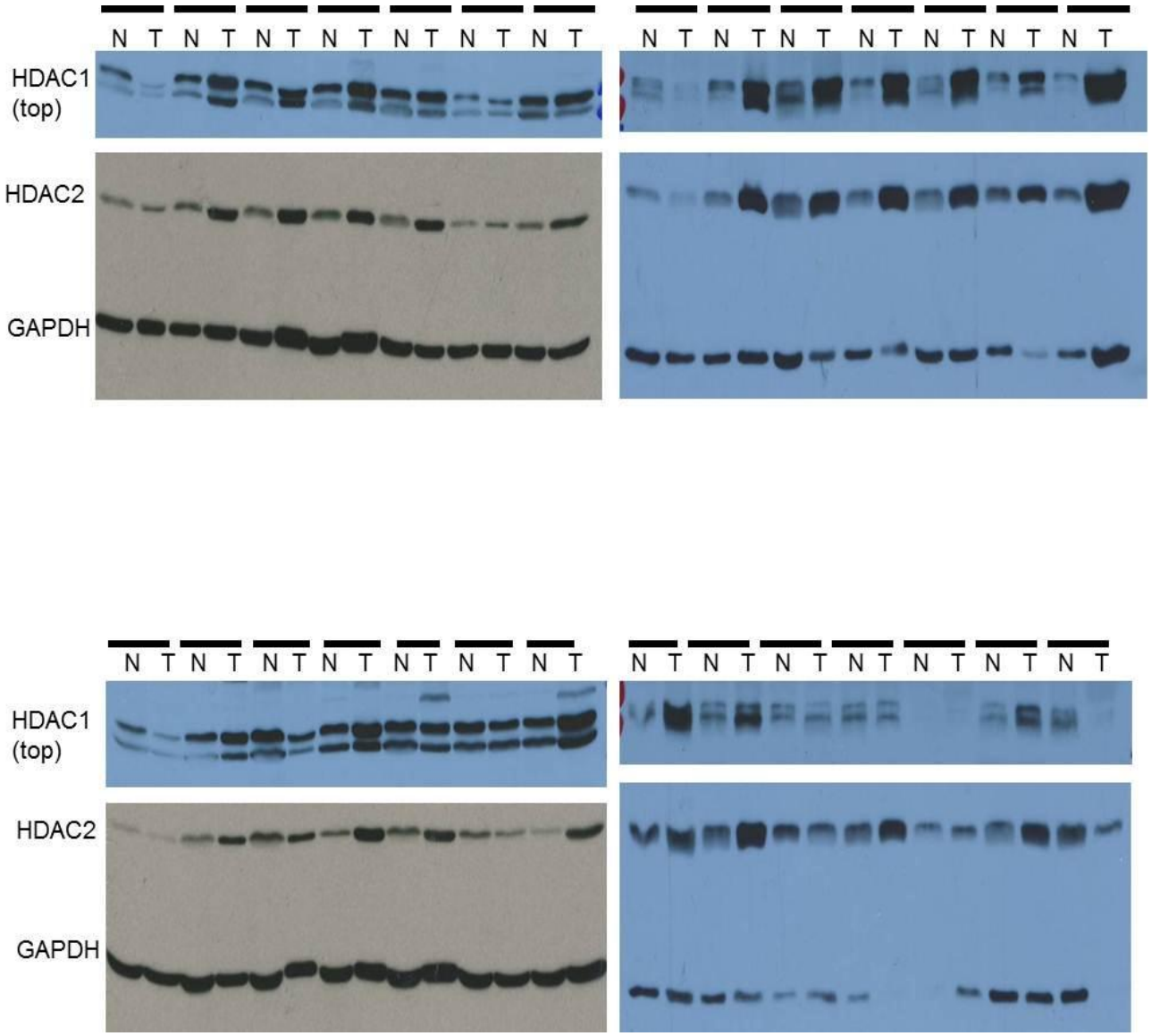
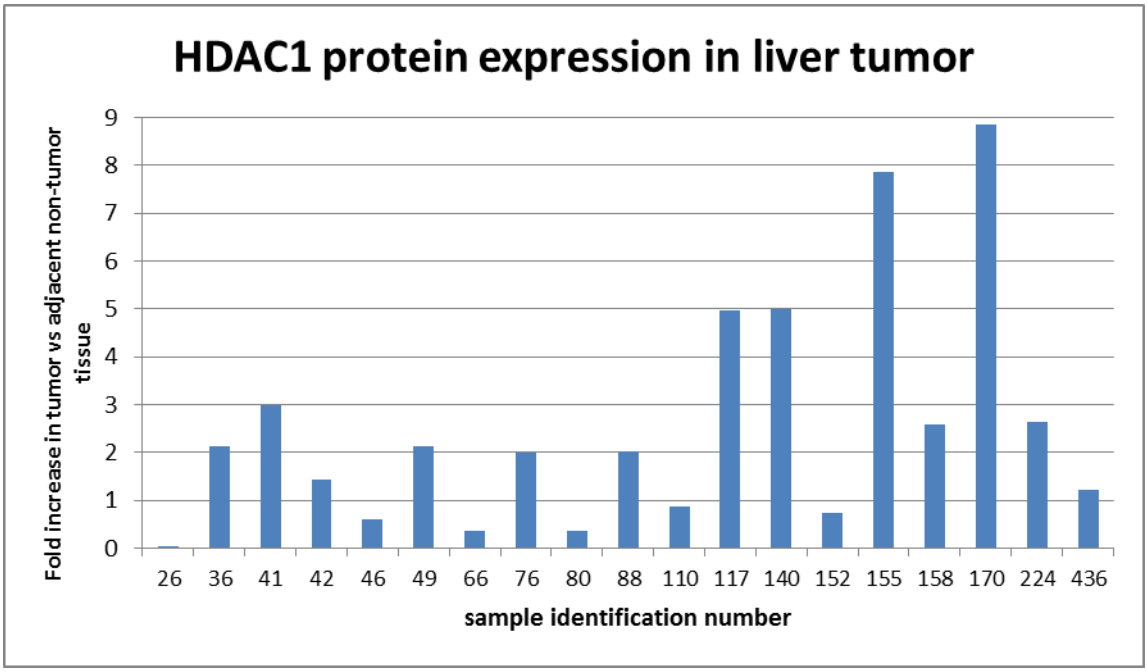


Figure 4.1 Protein expression of HDAC1 and HDAC2 are upregulated in liver tumor tissues compared to the matched adjacent normal tissues. Fifteen µg of protein from each sample was loaded and immunoblotted using anti-HDAC1 and anti-HDAC2 antibodies. GAPDH was used as a loading control. T represents the tumor tissue while N is the matched adjacent normal tissue.



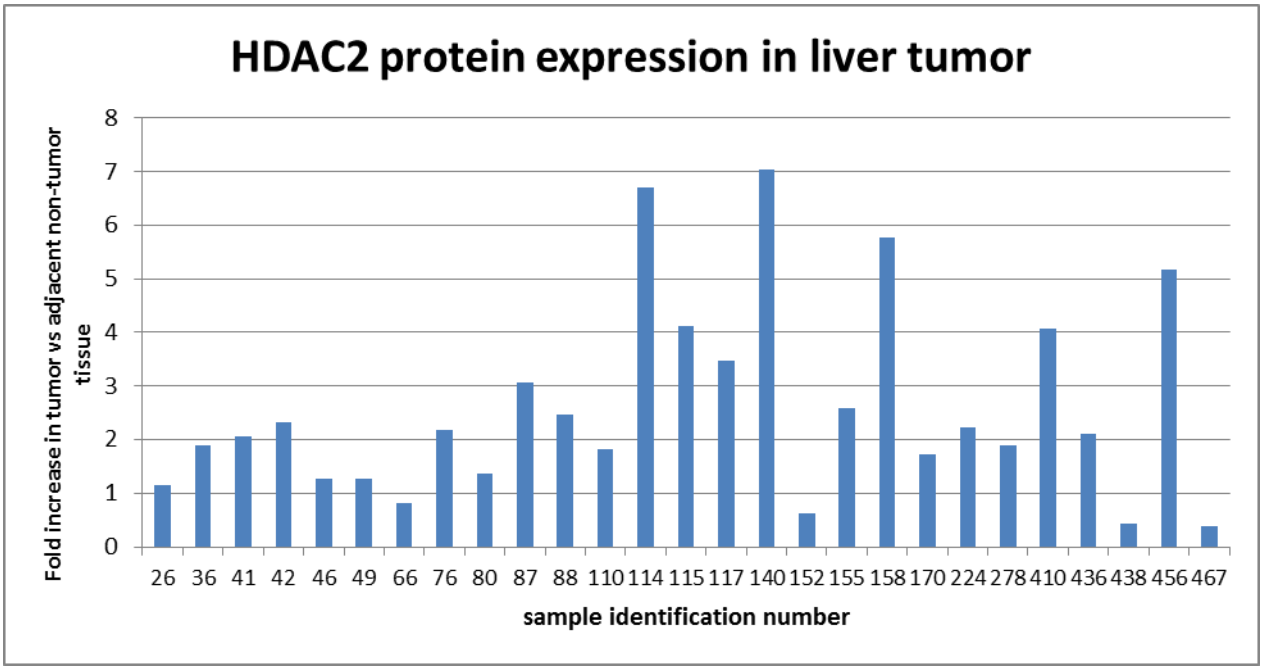


Figure 4.2 Densitometry to quantitate the fold increase in HDAC1 and HDAC2. The protein expression of HDAC1 (A) and HDAC2 (B) in liver tumor tissues was normalized to the matched adjacent normal tissues.

morphologically representative area on a bigger tissue block, as annotated by the pathologist. Immunohistochemistry was performed to examine the expression of HDAC1 and HDAC2 on these samples separately. Scoring was done based on the staining intensity of the hepatocytes' nuclei, with a score ranging from 0 to 3 (Figure 4.3). The number of samples with the various scores was tabulated in Table 4.1A. To find out if a matched sample pair has an upregulation of HDAC1 and HDAC2, the score for the non-tumor was subtracted from that of the tumor for each matched pair. Therefore, each matched pair would have a HDAC1 and HDAC2 index (T-N) ranging from -3 to 3. A negative index would indicate a downregulation of the protein in the tumor compared to the matched non-tumor, while a positive index would indicate an upregulation, and an index of 0 would indicate no change. Table 4.1B shows the number of samples with the various indices for HDAC1 and HDAC2. Majority of them have either no change or upregulation in HDAC1 and HDAC2. Of the 179 pairs, 58.1% (104 out of 179) has an upregulation of HDAC1, while 66.5% (119 out of 179) has an upregulation of HDAC2 (Table 4.1C). There was 46.4% (83 out of 179) with an upregulation in both HDAC1 and HDAC2; 14.5% (26 out of 179) of them with no change in both, but only 1.7% (3 out of 179) with a downregulation in both.

4.1.3 Correlation of HDAC1 and 2 expressions in hepatocellular carcinoma tissues with clinicopathological parameters

Clinicopathological factors were compared with the HDAC1 and HDAC2 expression in the tissue samples (Table 4.2). Among the parameters that were analyzed, upregulation in HDAC1 expression in the matched tumor sample was correlated with HBV status of the patient (p-value=0.039). Interestingly, patients who have cirrhosis or pre-operative treatment (embolization of tumor) were found to have

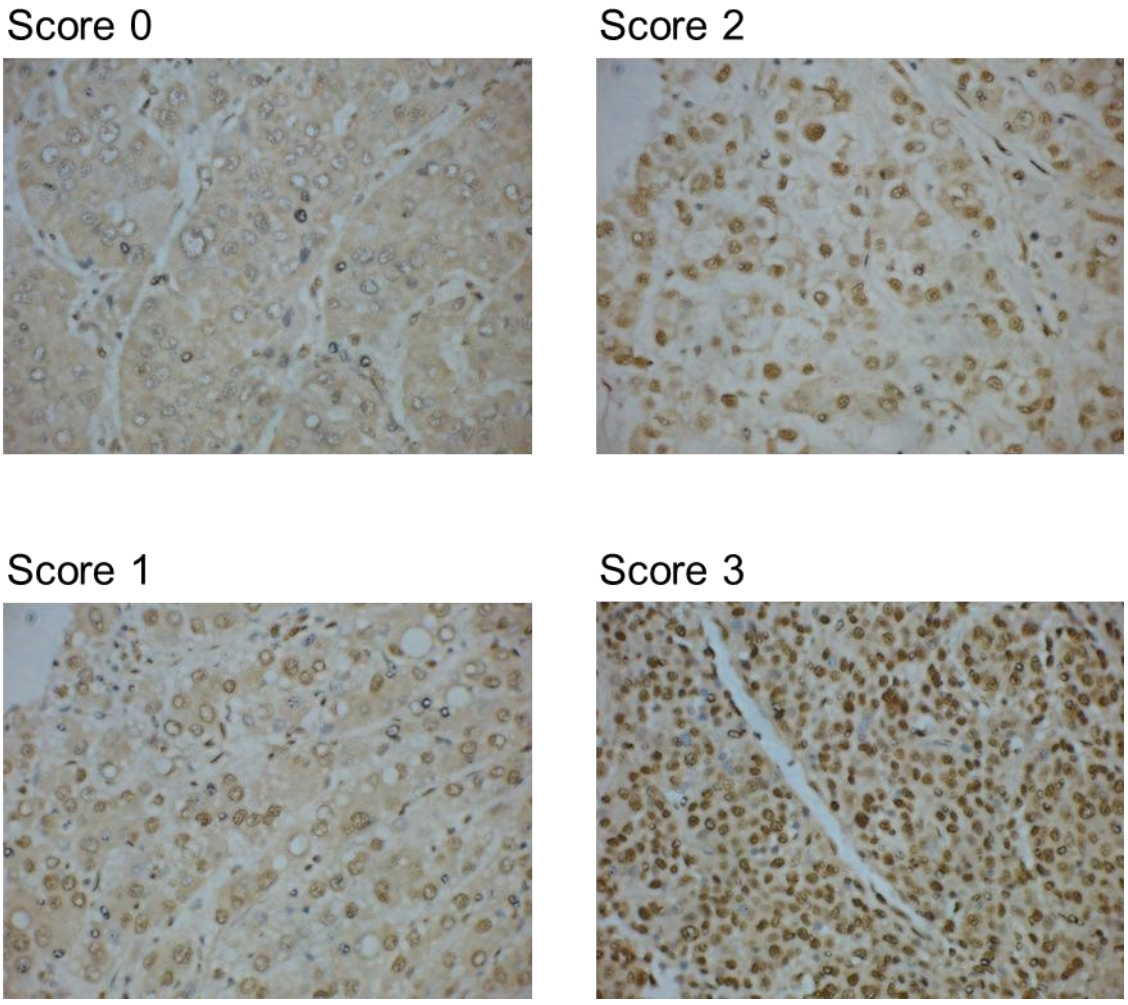


Figure 4.3 Immunohistochemical analysis of hepatocellular carcinoma tissue microarray. Representative examples of scoring performed on HDAC1 and HDAC2 staining. A tissue section with negative staining was given a score 0 while score 1 to 3 indicated increasing intensity of nuclear staining of the hepatocytes. Magnification of X400 is shown.

A

	HDAC1		HDAC2	
Grade	T	N	T	N
0	45	116	13	74
1	61	47	67	85
2	55	14	72	16
3	18	2	27	4

B

Index	HDAC1	HDAC2
-3	0	0
-2	0	1
-1	11	6
0	64	53
1	61	74
2	35	38
3	8	7

C

		HDAC2			
		Down	No chg	Up	Total
HDAC1	Down	3	6	2	11
	No chg	4	26	34	64
	Up	0	21	83	**104**
Total		7	53	**119**	179

Table 4.1 Summary of HDAC1 and 2 grading scores for HCC Tissue Microarray samples. A, The number of samples with a score of 0 to 3 for HDAC 1 and 2 in the tumor (T) and non-tumor (N) samples are shown. B, To obtain an index to whether the HDAC expression is upregulated, the score of the non-tumor sample was subtracted from that of the matched tumor sample. The table shows the number of samples with an HDAC1 or 2 index ranging from -3 to +3. A negative index indicates a downregulation whereas a positive index indicates an upregulated of HDAC1 or 2 in the matched tumor and non-tumor sample. An index of zero indicates no change in HDAC1 or 2 expression between the matched tumor and non-tumor sample. C, This crosstable shows the number of samples with either downregulated, no change, or upregulated HDAC1 and 2.

Table 4.2 Comparison of clinical parameters between matched samples that have downregulated, no change, or upregulated HDAC1 and HDAC2.

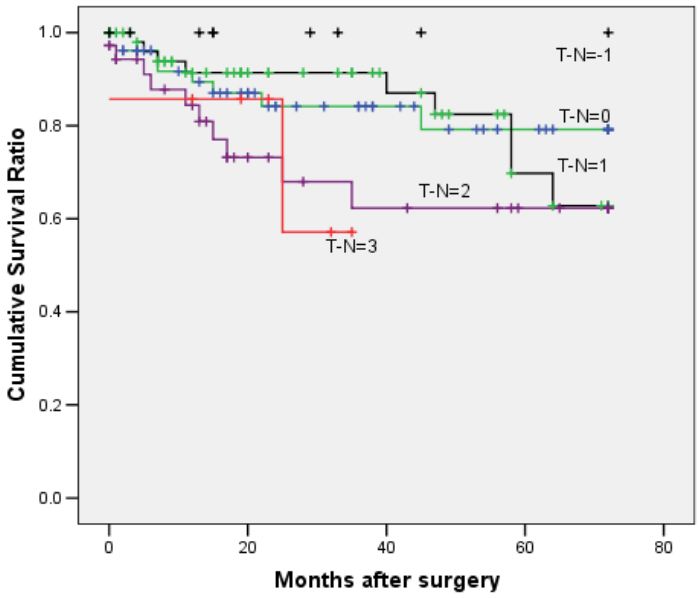
	HDAC1			HDAC2		
	Down	No change	UP	Down	No change	UP
Mean age (years)	54.6+6.5	59.9+13.8	57.3+13.8	62.5+9.3	59.6+11.4	57.1+14.5
Gender	F=2 M=9	F=5 M=59	F=24 M=80	F=1 M=6	F=7 M=46	F=23 M=96
Race	Chinese=8	Chinese=52	Chinese=80	Chinese=4	Chinese=45	Chinese=91
	Others=2	Others=12	Others=22	Others=3	Others=8	Others=27
Smoking	4/6=66.6%	20/48=41.7%	28/79=35.4%	3/5=60%	21/41=51.2%	28/87=32.2%
Alcohol	5/7=71.4%	20/49=40.8%	30/82=36.6%	4/6=66.7%	19/42=45.2%	22/80=27.5%
Inflammation	3/11=27.3%	12/64=18.8%	16/104=15.4%	2/7=28.6%	13/53=24.5%	16/119=13.4%
Cirrhosis	10/11=90.9%	31/64=48.4%	49/104=47.1%	5/7=71.4%	27/53=50.9%	58/119=48.7%
Fibrosis	2/11=18.2%	9/64=14.1%	23/104=22.1%	1/7=14.3%	14/53=26.4%	19/119=16.0%
Tumor size (cm)	5+3.8	7.2+4.5	6.2+4.1	5.2+3.2	6.5+4.7	6.2+3.9
Group Stage	I=5/9 (55.6%)	I=24/53 (45.3%)	I=48/95 (50.5%)	I=3/7 (42.9%)	I=19/45 (42.2%)	I=55/105 (52.4%)
	II=1/9 (11.1%)	II=19/53 (35.8%)	II=29/95 (30.5%)	II=1/7 (14.3%)	II=17/45 (37.8%)	II=31/105 (29.5%)
	III=3/9 (33.3%)	III=10/53 (18.9%)	III=18/95 (18.9%)	III=3/7 (42.9%)	III=9/45 (20%)	III=19/105 (18.1%)
Histology grade	P=1/10 (10%)	P=9/54 (16.7%)	P=15/90 (16.7%)	P=1/7 (14.3%)	P=8/46 (17.4%)	P=16/101 (15.8%)
	M=5/10 (50%)	M=31/54 (57.4%)	M=60/90 (66.7%)	M=5/7 (71.4%)	M=24/46 (52.2%)	M=67/101 (66.3%)
	W=4/10 (40%)	W=14/54 (25.9%)	W=15/90 (16.7%)	W=1/7 (14.3%)	W=14/46 (30.4%)	W=18/101 (17.8%)
Lymph node invasion	1/7=14.3%	5/23=21.7%	8/54=14.8%	2/5=40%	4/22=18.2%	8/57=14%
Vascular invasion	2/8=25%	23/54=42.6%	35/89=39.3%	2/6=33.3%	21/46=45.7%	37/99=37.4%
HBV	7/9=77.8%	30/55=54.5%	69/97=71.1%	3/7=42.9%	31/47=66%	72/107=67.3%
HCV	1/8=12.5%	3/53=5.7%	5/97=5.2%	1/7=14.3%	1/46=2.2%	7/110=6.4%
Pre-Op Treatment	5/9=55.6%	11/56=19.6%	16/96=16.7%	1/7=14.3%	11/46=23.9%	20/108=18.5%

downregulated HDAC1 expression in their tumor with p-value of 0.021 and 0.020 respectively. Chi-square test was used in the above analysis.

4.1.4 Correlation of HDAC1 and 2 expressions with patient survival rates

Kaplan-Meier analysis was used to study how the HDAC1 and HDAC2 expression affects survival rates of patients. Using the HDAC1 and HDAC2 indices, which are measures of the respective HDAC expressions in the tumor compared to the matched non-tumor sample (T-N), the cumulative survival ratio of the total population of samples was plotted over 6 years (Figure 4.4). There is a general trend that patients with higher HDAC1 index, indicating a higher upregulation of HDAC1 in the tumor compared to the matched non-tumor sample, have shorter survival time (Figure 4.4A). However, such trend was not observed when HDAC2 index was used for the same analysis (Figure 4.4B). To simplify the analysis, the matched samples were further categorized into low expression (T-N index equals or less than 1) or high expression (T-N index more than 1) for HDAC1 and HDAC2 separately. The cumulative survival ratio of the total population of samples was plotted over 6 years (Figure 4.5). Patients with higher HDAC1 index have a significantly poorer prognosis compared to those with a lower index ($p=0.037$). Those with a higher HDAC2 index showed slightly poorer prognosis from the 2nd year onwards but it was overall not statistically significant ($p=0.511$). To control for other parameters which may also affect survival of liver cancer patients, a Cox Regression (Table 4.3) was done and higher HDAC1 expression remained as a significant prognosis factor for poorer survival ($p=0.015$). As such, HDAC1 upregulation appeared to be a prognosis factor for survival of HCC patients.

A



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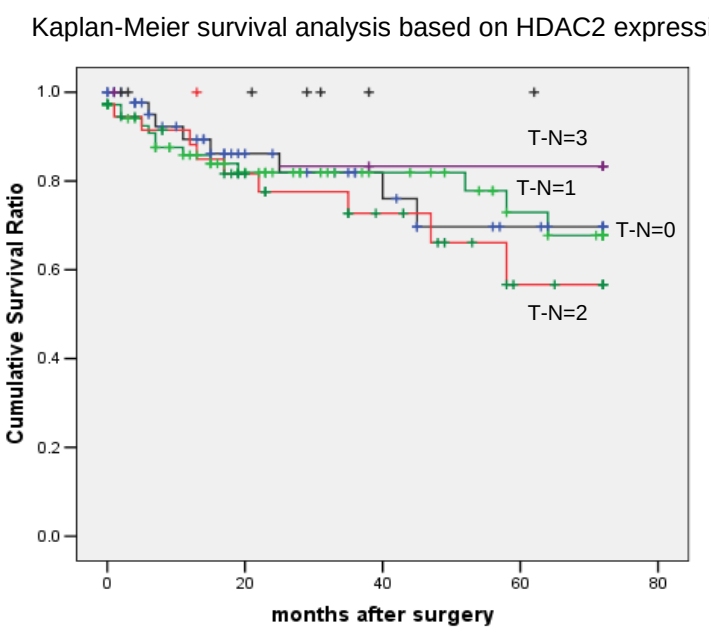


Figure 4.4 Kaplan-Meier curve to compare survival rate of patients with different HDAC indices. A, Patients with a higher HDAC1 index generally has a lower survival rate than those with a lower HDAC1 index (p=0.149). B, There is no well-defined trend between the HDAC2 index and patient survival rates.

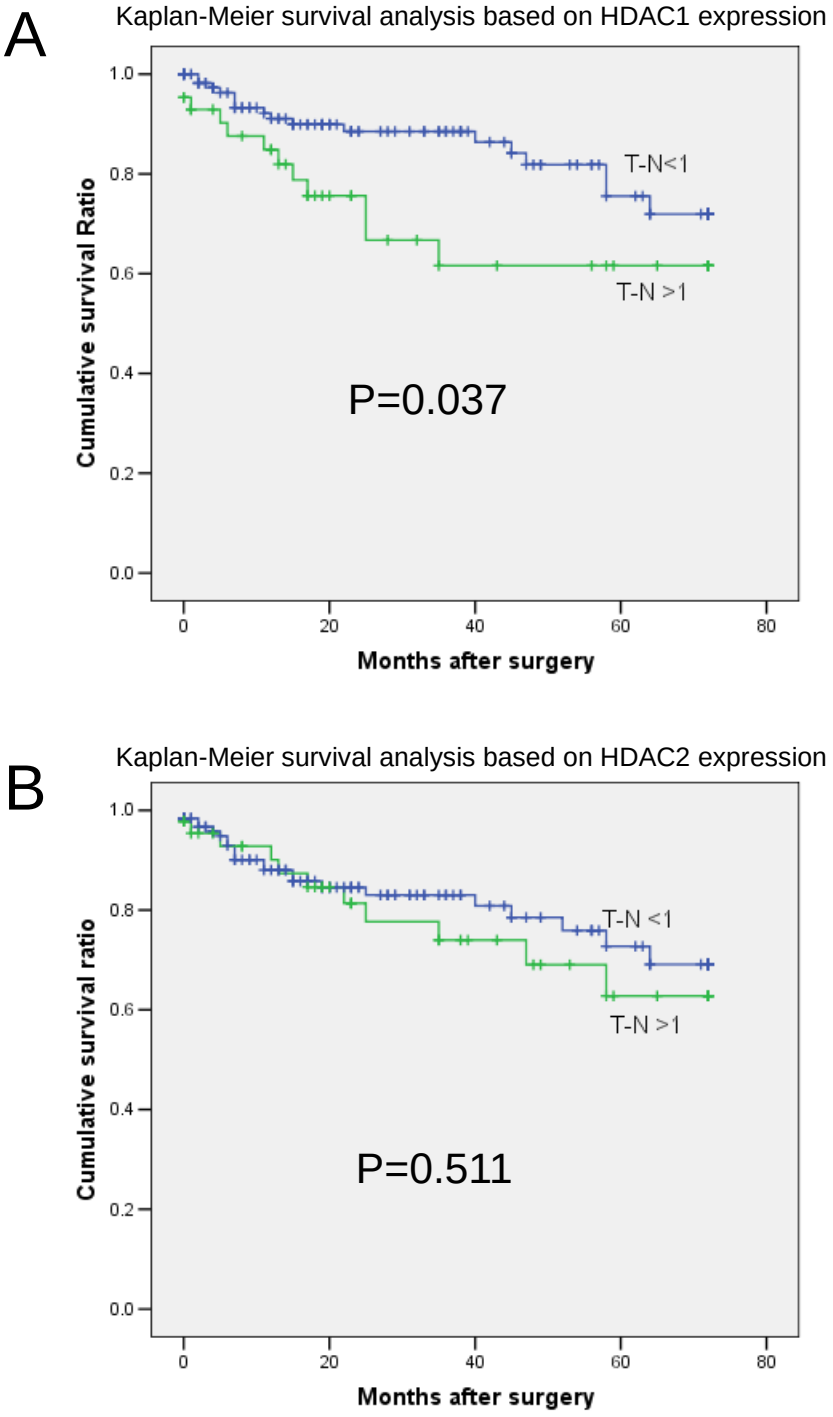


Figure 4.5 Kaplan-Meier curve to compare survival rate of patients with a HDAC index of less than or equal to 1, against those with an index of more than 1. A, Patients with a higher HDAC1 index has a lower survival rate than those with a lower HDAC1 index (p=0.037). B, There is no statistical significance in the survival rates of patient with higher or lower HDAC2 index (p=0.511).

Table 4.3 Summary of univariate and multivariate cox regression analysis of patient survival.

	Hazard Ratio	p-value
HDAC1	2.148	**0.043**

	Hazard Ratio	p-value
HDAC2	1.273	**0.513**

	Hazard Ratio	*p-value*
HDAC1	4.782	**0.015**
histology grade		0.533
histology grade (1)	1.241	0.864
histology grade (2)	0.556	0.688
HBV	0.885	0.856
HCV	3.496	0.355
cirrhosis	2.958	0.124
symptoms	0.929	0.918
vascular invasion	3.93	0.088
inflammmation	1.375	0.709
Age	1.043	0.067
Gender	1.112	0.897
tumor size	1.113	0.267
staging analysis		0.338
staging analysis(1)	2.439	0.256
staging analysis(2)	3.564	0.144
log AFP	2.292	0.003

	Hazard Ratio	*p-value*
HDAC2	0.853	**0.773**
histology grade		0.595
histology grade (1)	2.027	0.57
histology grade (2)	1.116	0.938
HBV	1.039	0.949
HCV	5.98	0.161
cirrhosis	2.642	0.142
symptoms	0.857	0.808
vascular invasion	5.618	0.029
inflammmation	1.048	0.957
Age	1.037	0.063
Gender	0.429	0.187
tumor size	1.145	0.11
staging analysis		0.885
staging analysis(1)	1.334	0.718
staging analysis(2)	1.503	0.623
log AFP	2.094	0.004

4.1.5 Expressions of HDAC1 and 2 in various colon and liver cancer cell lines

The protein expression of HDAC1 and HDAC2 was examined in various colon (HT29, RKO, and HCT116) and liver (HEP3B, HEPG2, PLC5) cancer cell lines. As shown in Figure 4.6, both HDAC1 and HDAC2 were expressed in all the cell lines tested.

4.2 Verification of efficiency and specificity of siRNA against HDAC1 and 2

A knockdown strategy was used to study the functions of HDAC1 and HDAC2 in cells. Because of the high homology between HDAC1 and HDAC2, it is important to ensure that the siRNA designed to silence the expression of HDAC1 and HDAC2 are specific.

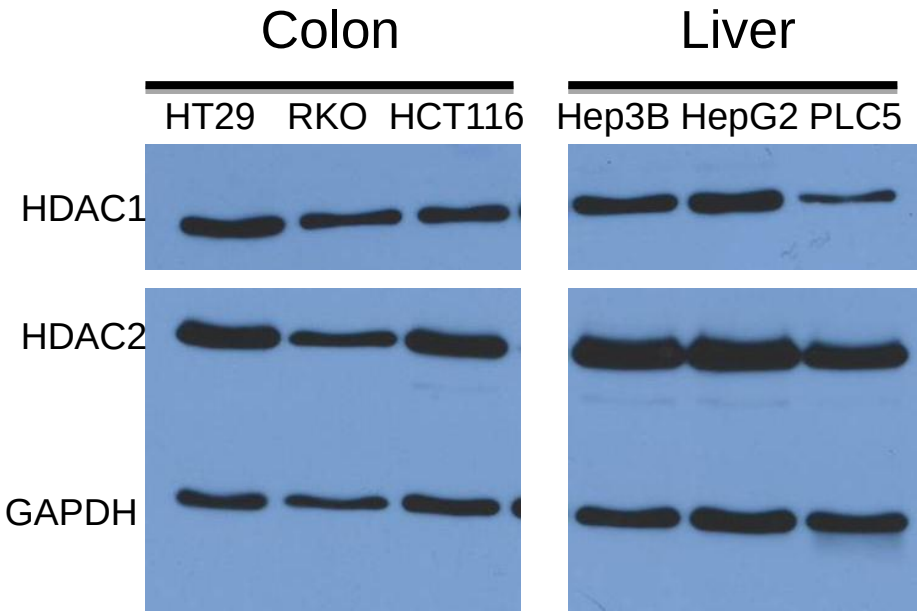


Figure 4.6 Comparison of HDAC1 and 2 protein expression among the various colon and liver cancer cell lines. Protein was extracted from the human colorectal cancer cell lines HT29, RKO and HCT116 as well as the liver cancer cell lines HEP3B, HEPG2, and PLC5. Western blot was performed using anti-HDAC1 and anti-HDAC2 antibodies. GAPDH was used as loading control.

After transfecting HEP3B cells with the individual siRNA sequences individually, RNA was extracted and the mRNA expression of HDAC1 and HDAC2 was quantified using real-time RT-PCR. Results showed that the siRNA sequences against HDAC1 can reduce HDAC1 expression without reducing that of HDAC2, and vice versa (Figure 4.7). There was at least 8-fold decrease in gene expression when the individual siRNA were used for the specific HDAC. In other words, the siRNAs were both specific and efficient in knocking down HDAC1 and HDAC2. Similar level of knockdown was achieved when the sequenced were mixed to knockdown HDAC1 and 2 together. Western blot was used to verify that the knockdown specificity and efficiency was demonstrated at the protein level as well. The siRNA was specific and efficient when used separately (Figure 4.8A) or when the 3 sequences were pooled to be used together (Figure 4.8B). Subsequently, the siRNA were pooled together to knockdown HDAC1 or HDAC2 or both.

It is interesting to note that while the siRNA against HDAC1 and HDAC2 was specific in knocking down the respective HDACs, the knockdown of one seemed to increase the protein expression of the other (Figure 4.8). This phenomenon was consistent in both the HCT116 and HEP3B cells.

4.3 Effects of HDAC1 and 2 knockdown on cancer cells survival

4.3.1 Reduction of colony formation after knockdown of both HDAC1 and 2 in different cell lines

The expression of HDAC1 and HDAC2 were knocked down individually or together in 3 liver cancer cell lines and 1 colorectal cancer cell line: HEP3B, HEPG2, PLC5, and HCT116. After transfection, the cells were allowed to recover overnight before being replated at low density to test for their ability to form colonies. Figure

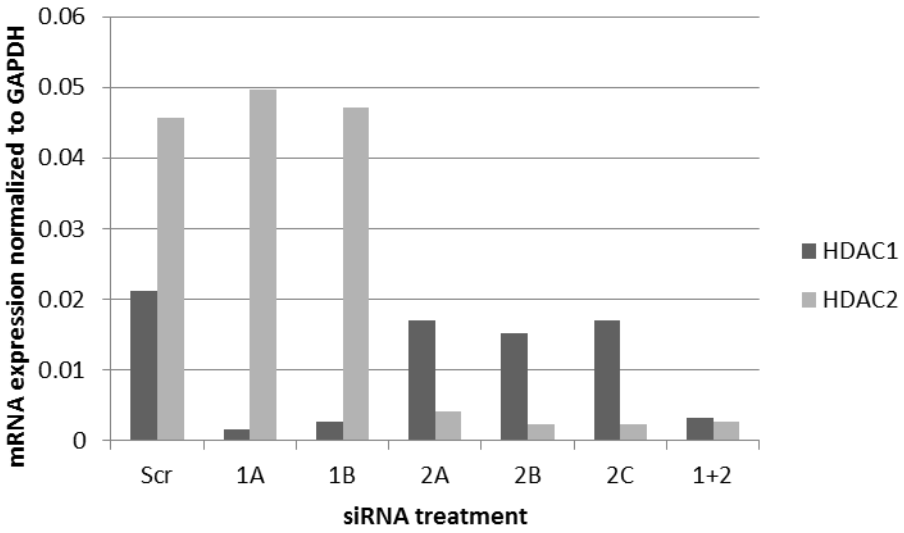


Figure 4.7 Quantitative real time RT-PCR to show efficiency and specificity of HDAC1 and HDAC2 knock-down. HEP3B cells were transfected with either HDAC1 or HDAC2 specific siRNA for 72 hours. Cells were harvested and RNA extracted and used for quantitative real time RT-PCR, done in replicates. The sequences specific for HDAC1 (1A and 1B) resulted in reduced RNA expression of HDAC1 compare to the non-silencing control (Scr) but not HDAC2, and the sequences specific for HDAC2 (2A, 2B, and 2C) resulted in reduced RNA expression of HDAC2 compare to the non-silencing control (Scr) but not HDAC1. Data is from duplicates and is representative of 3 independent experiments.

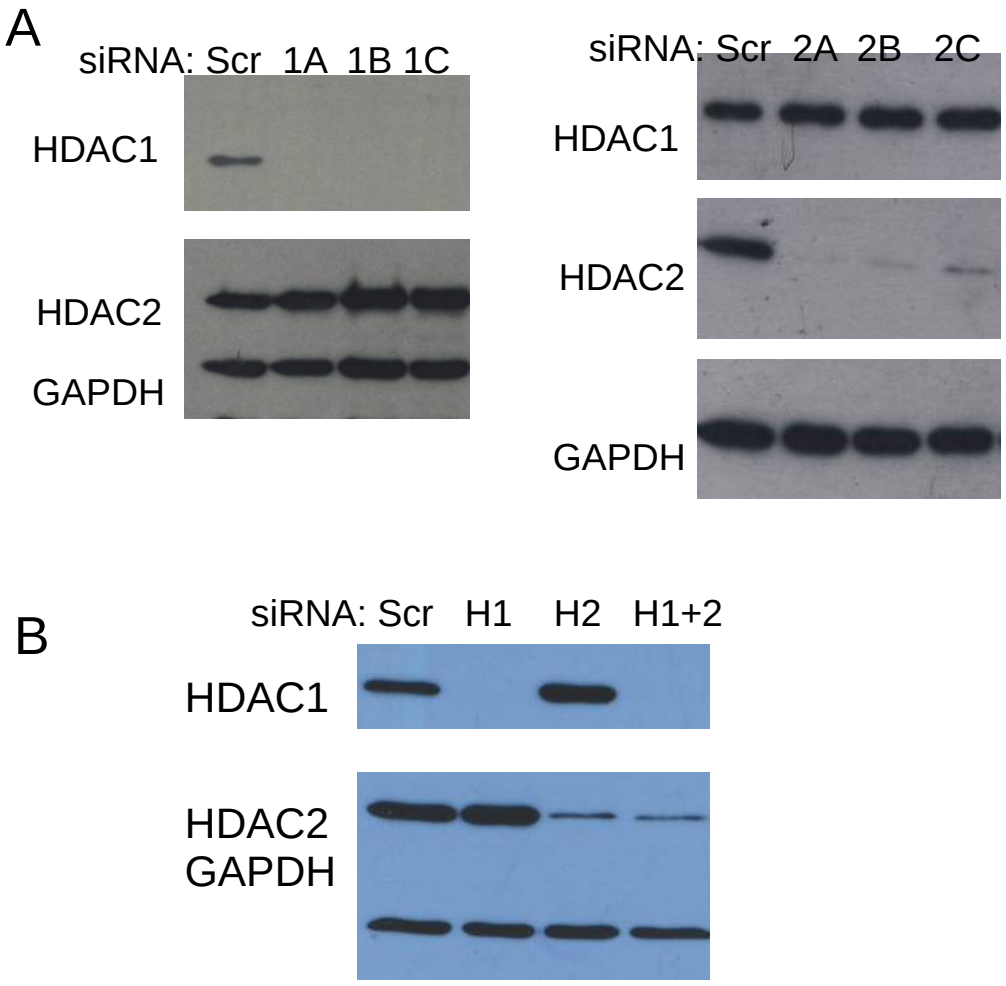


Figure 4.8 Western blot to show specificity and efficiency of HDAC1 and HDAC2 knockdown. A, HCT116 cells were transfected with either HDAC1 or HDAC2 specific siRNA (3 sequences for each HDAC) for 72 hours. Cells were harvested and total protein extracted. Western blot was done using antibody for either HDAC1 or HDAC2. The 3 sequences specific for HDAC1 (1A, 1B, and 1C) resulted in reduced protein expression of HDAC1 compare to the non-silencing control (Scr) but not HDAC2, and the 3 sequences specific for HDAC2 (2A, 2B, and 2C) resulted in reduced protein expression of HDAC2 compare to the non-silencing control (Scr) but not HDAC1. There was a slight upregulation of HDAC1 when HDAC2 was silenced, and vice versa. GAPDH was used as loading control. B, HEP3B cells were transfected with a mixture of 3 siRNA sequences against HDAC1 (H1), or HDAC2 (H2), or 6 siRNA sequences against both HDAC1 and HDAC2 (H1+2), or the non-silencing control (Scr) for 72 hours before being harvested for protein for Western blot using HDAC1 and HDAC2 specific antibodies. There was a slight upregulation of HDAC1 when HDAC2 was silenced, and vice versa. GAPDH was used as loading control.

4.9 shows the western blot to verify the knockdown of HDAC1 and/or HDAC2 proteins. Figure 4.10 shows the representative images of colonies formed after treatment with HDAC1 or/and HDAC2 siRNA, the non-silencing siRNA control (Scr) or the untransfected control (Ctrl). The number of colonies in each well was counted by ImageJ software and the quantified data is shown in Figure 4.11. In all the 4 cell lines tested, the number of colonies formed was reduced, to varying degrees, when both HDAC1 and 2 were knocked down together. However, in all the cell lines tested, this effect was not observed when HDAC1 and 2 were silenced individually.

4.3.2 Reduction in cell proliferation over 6 days after knockdown of both HDAC1 and 2

To observe the growth curve of cells after knocking down HDAC1 and HDAC2, HEP3B cells were treated with HDAC1 or/and HDAC2 siRNA, the non-silencing siRNA control (Scr) or the untransfected control (Ctrl) and replated in 96-well dishes. WST-1 assay was performed to assess cell number as a surroagate measure of proliferation of these cells over 6 days. Figure 4.12 shows that the growth of the HEP3B cells with both HDAC1 and 2 silenced stopped growing after 72 hours, and the cell number started to decrease from that point until 120 hours post-transfection. On the other hand, individually knocking down HDAC1 or HDAC2 did not have significant effect on the cell proliferation over time.

4.3.3 Cell cycle profile analysis showed increase in apoptosis in cells after knockdown of HDAC1 and 2

To account for the reduced colony formation and proliferation of the cells after knocking down HDAC1 and 2 expressions, we used flow cytometry to examine their cell cycle profiles (Figure 4.13). Gating was done to quantify the percentage of the cell population in each phase of the cell cycle: subG1, G1, S, and G2M (Figure 4.14).

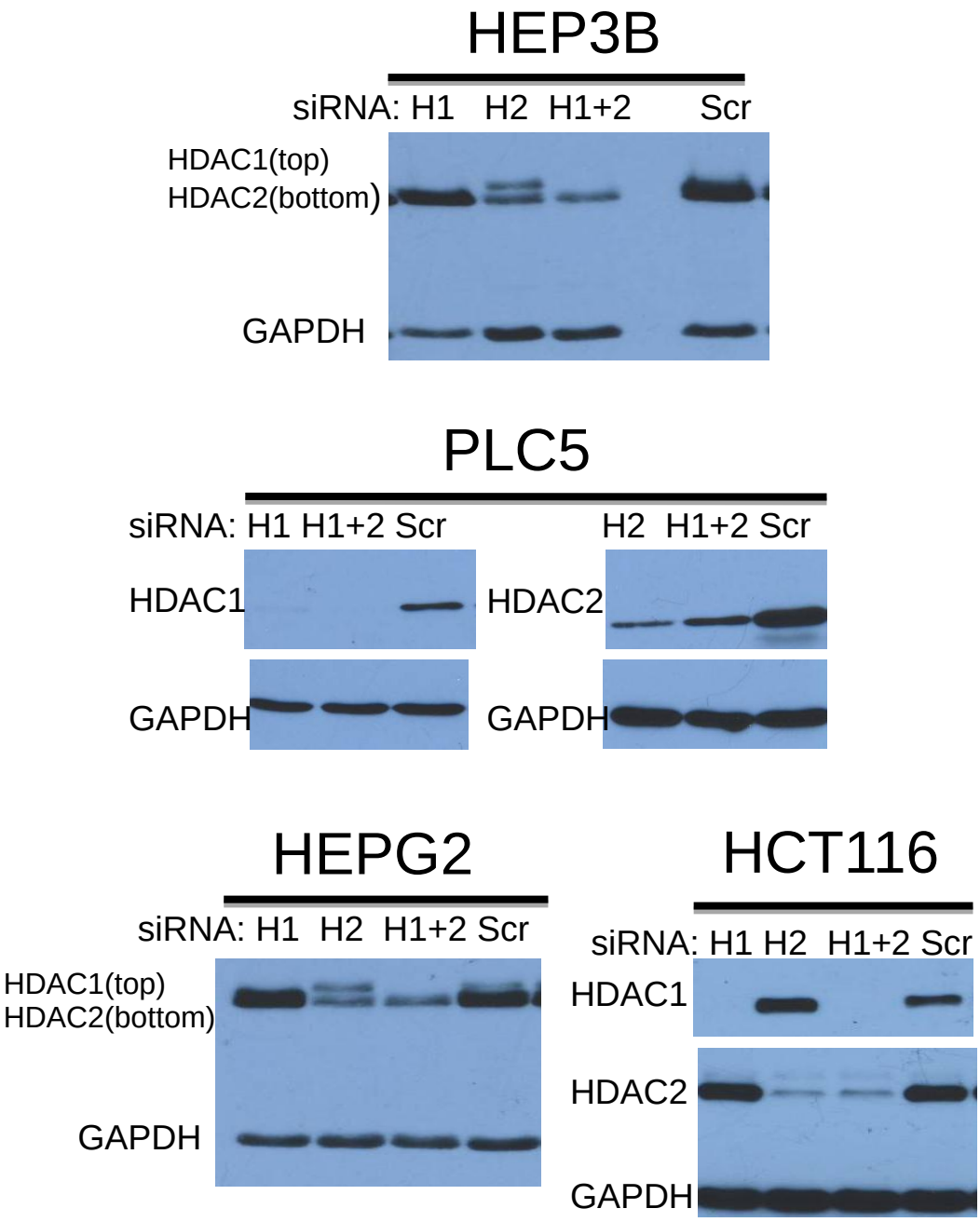


Figure 4.9 Knockdown of protein expression of HDAC1 or/and HDAC2 in HEP3B, HEPG2, PLC5, and HCT116 cells. Cells were transfected with HDAC1 siRNA (H1), or HDAC2 siRNA (H2), or both (H1+2), or non-silencing control siRNA (Scr). After 72 hours, cells were harvested for protein and Western blot using HDAC1 and HDAC2 specific antibodies was done. GAPDH was used as loading control.

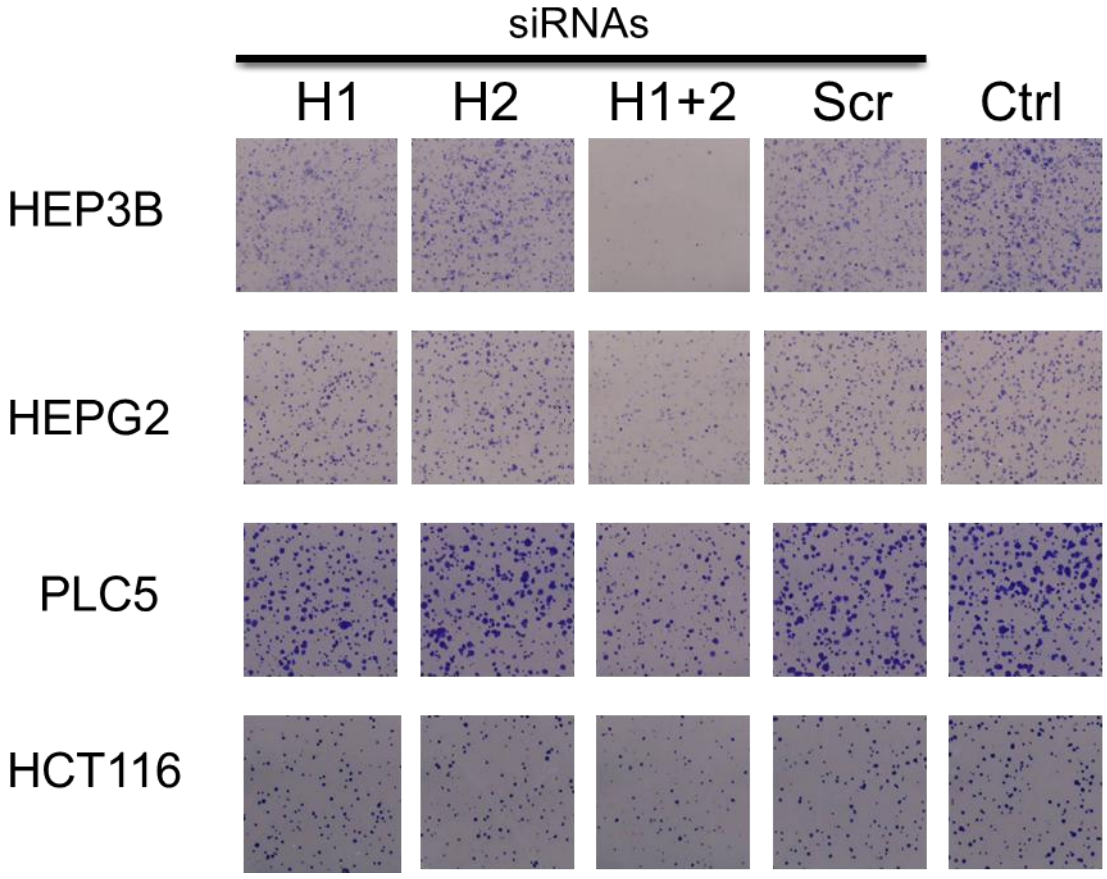


Figure 4.10 Effect of knocking down HDAC1 or/and HDAC2 in HEP3B, HEPG2, PLC5, and HCT116 cells. Cells were transfected with HDAC1 siRNA (H1), or HDAC2 siRNA (H2), or both (H1+2), or non-silencing control siRNA (Scr) or untransfected (Ctrl). They were allowed to recover overnight before being counted and replated at low density. After 10 days, the colonies were stained with crystal violet and the wells were imaged. The knockdown of both HDAC1 and 2 together is required to reduce colony formation. Representative data from 3 independent experiments.

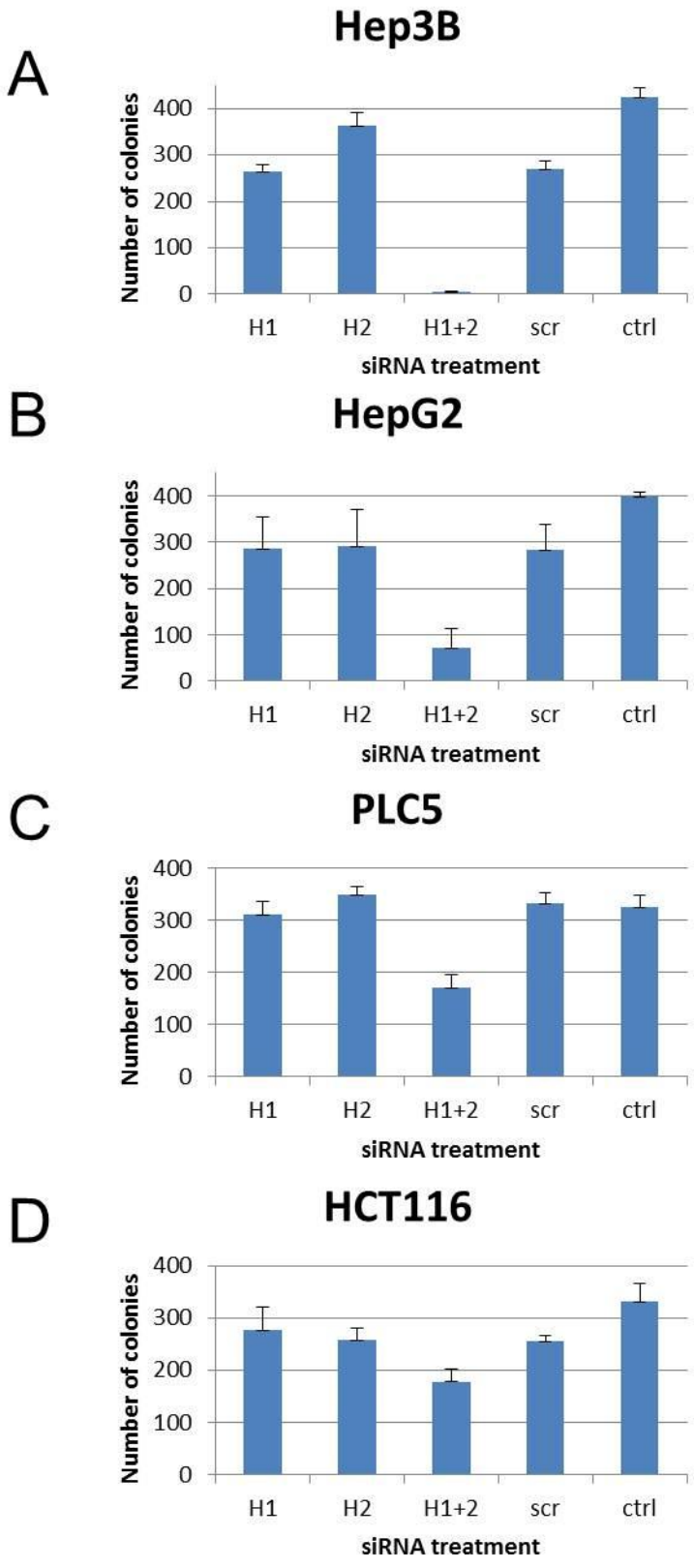


Figure 4.11 Quantification of colony formation in HEP3B. (A), HEPG2 (B), PLC5 (C), and HCT116 (D) cells after knocking down HDAC1 or/and HDAC2. The average number of colonies in each of the triplicate wells were plotted against the treatment. Error bars showed standard deviations. The knockdown of both HDAC1 and 2 together is required to reduce colony formation.

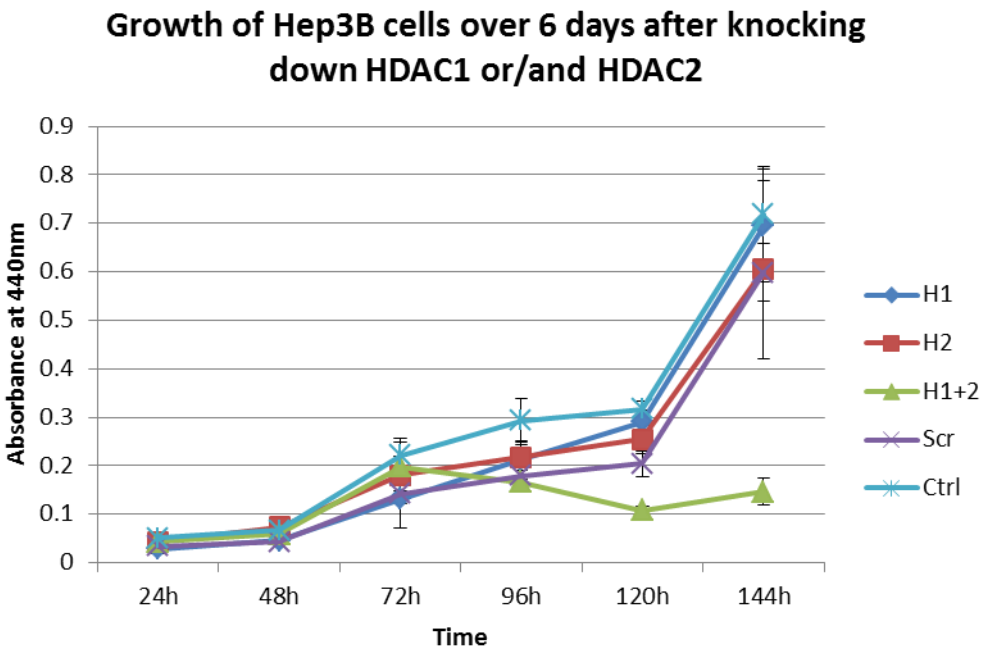


Figure 4.12 WST-1 assay showed that knocking down HDAC1 and 2 can reduce cell growth over time. HEP3B cells were transfected with HDAC1 siRNA (H1), or HDAC2 siRNA (H2), or both (H1+2), or non-silencing control siRNA (Scr) or untransfected (Ctrl). They were allowed to recover overnight before being counted and replated in 5 replicates into six 96-well dishes. Each plate was taken out for WST-1 assay at the various time points. Error bars showed standard deviations.

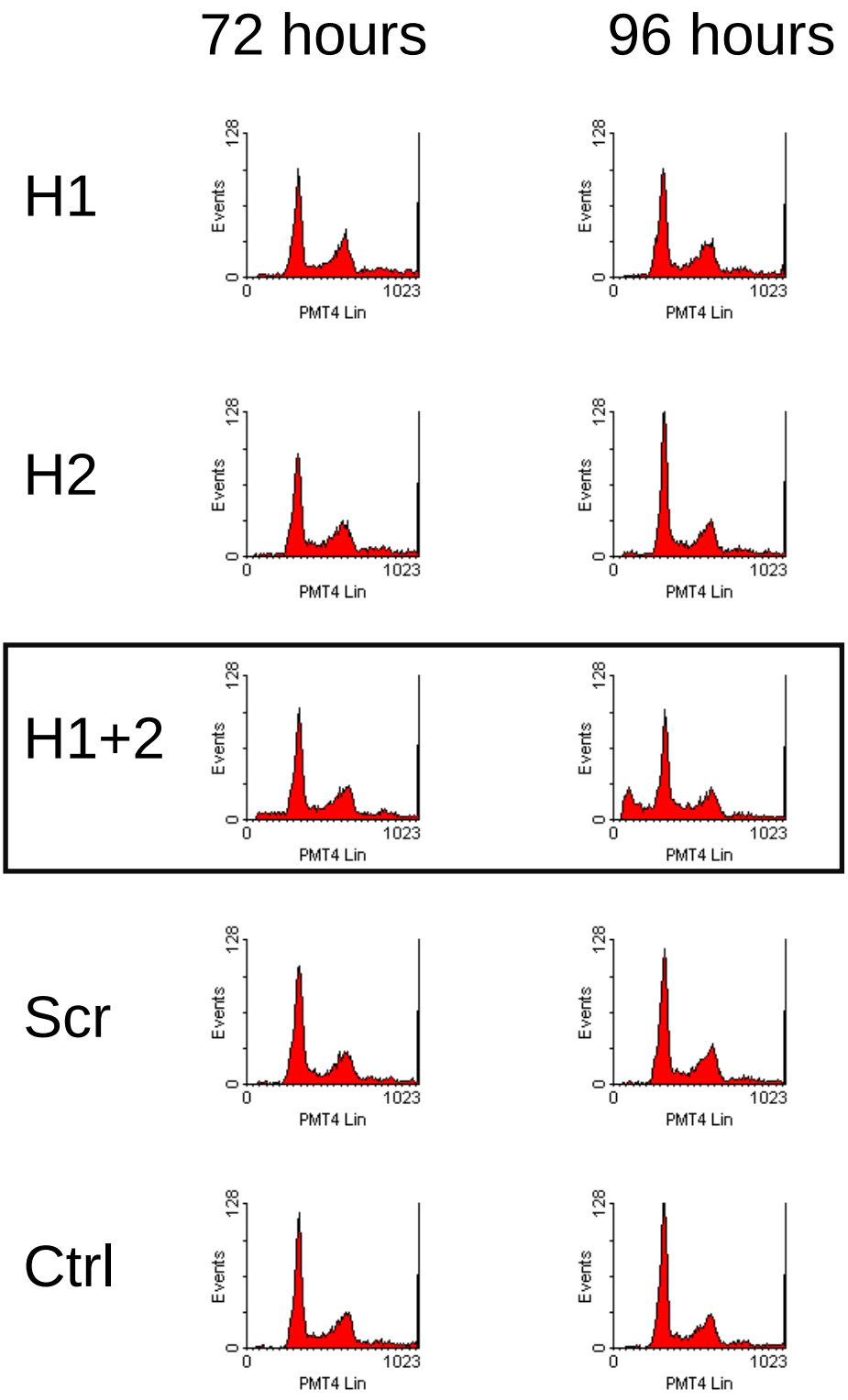


Figure 4.13 Cell cycle analysis of HEP3B cells. Histograms to show the cell cycle profiles of HEP3B cells at 72 hours and 96 hours after siRNA treatment to knockdown HDAC1 or/and HDAC2. HEP3B cells were transfected with HDAC1 siRNA (H1), or HDAC2 siRNA (H2), or both (H1+2), or non-silencing control siRNA (Scr) or untransfected (Ctrl). The cell cycle profile was changed when both HDAC1 and 2 were silenced together.

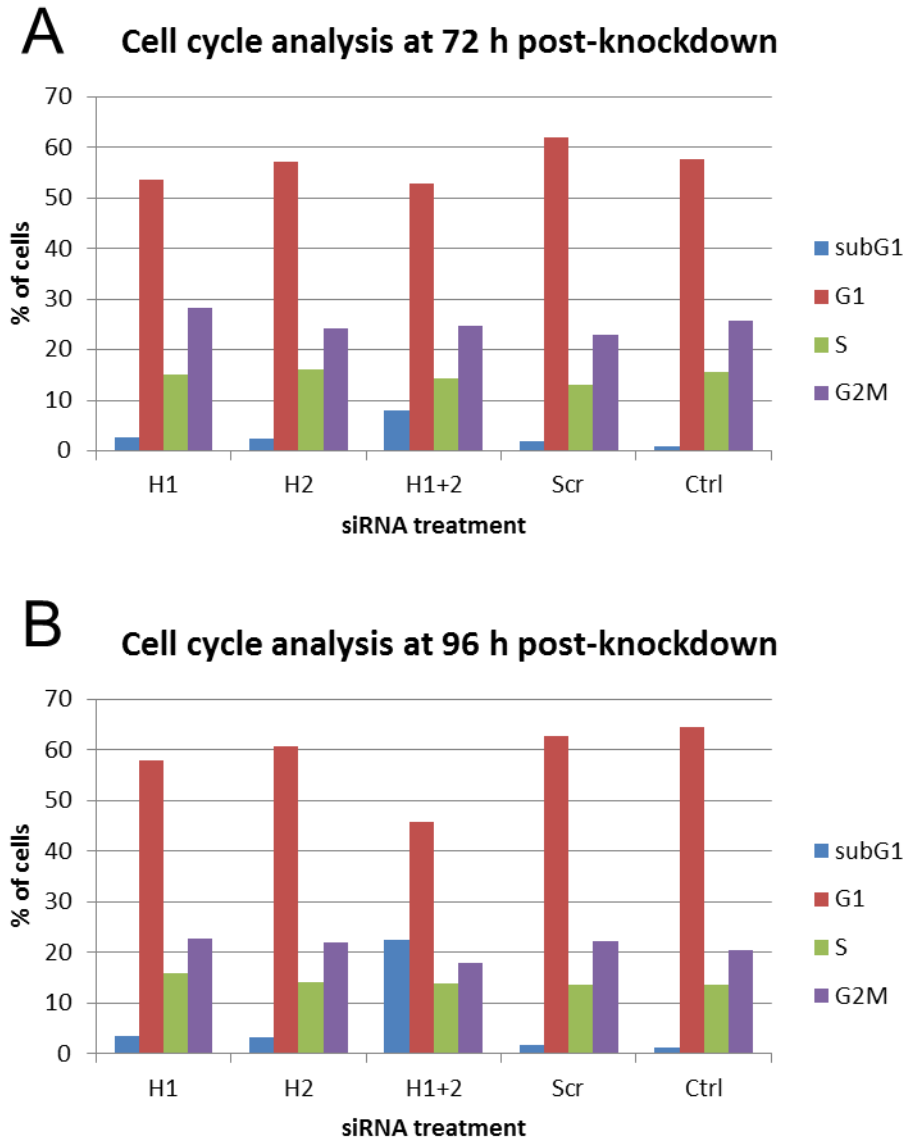


Figure 4.14 Quantification of the percentages of cells in each phase of the cell cycle at 72 hours (A) and 96 hours (B) after siRNA treatment to knockdown HDAC1 or/and HDAC2 in HEP3B cells. The knockdown of both HDAC1 and 2 together increased the percentage of cells in the subG1 and reduced the percentage of cells in the G1 phase of the cell cycle. Data is representative of 3 independent experiments.

The cell cycle profiles of HEP3B cells transfected with HDAC1 or HDAC2 siRNA individually, or the non-silencing siRNA (Scr), or the untransfected control, remained unchanged after 72 and 96 hours. For cells that had both HDAC1 and HDAC2 knocked down, the percentage of their subG1 fraction increased while that of the G1 decreased. This observation was more prominent at 96 hours than 72 hours post-transfection. A closer examination of the subG1 fractions of the cell population, which represents the apoptotic cells, showed that the percentage of cells increased from 8 % at 72 hour to 22.5% at 96 hours post-transfection, whereas it was only about 2% in the control (Figure 4.15). This is consistent with the time points when a drop in the growth curve was seen as described in the previous section.

4.3.4 Changes in expression of apoptotic proteins after knockdown of HDAC1 and 2

Western blot was done to check the efficiency of knockdown as well as the expression of various proteins involved in apoptosis. Figure 4.16 shows that the knockdown was sustained throughout the period from 72 hours to 120 hours post-transfection. There was cleavage of caspase 3 from 96 hours to 120 hours post-transfection. This caspase 3 was active as indicated by the cleavage of its substrate PARP. Another apoptotic protein BCL2 remained unchanged.

4.4 Mechanisms for reduced cell survival after knockdown of HDAC1 and 2

4.4.1 Synergistic reduction in global HDAC activity after knockdown of HDAC1 and 2

We used a HDAC activity assay to measure the global HDAC activity in the cell after knocking down HDAC1 or/and HDAC2. Figure 4.17A shows that while silencing HDAC2 has no effect on the global HDAC activity of HEP3B cells,

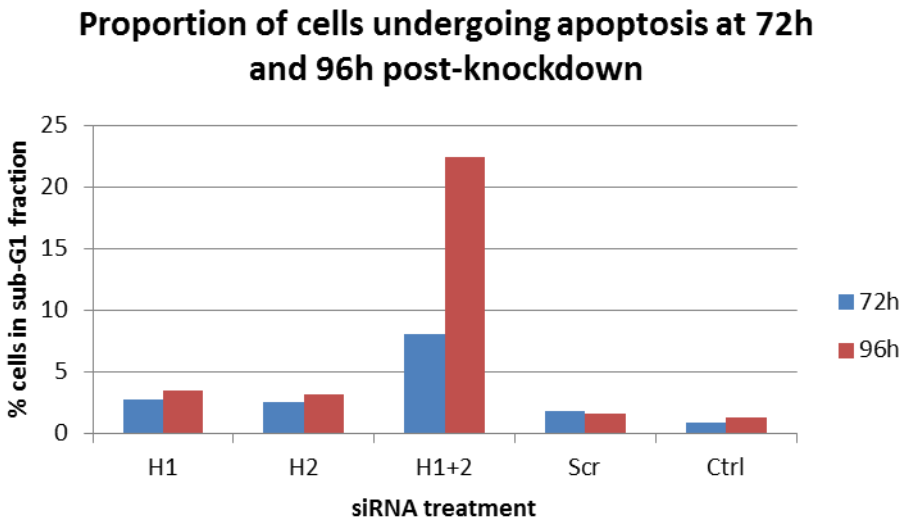


Figure 4.15 Apoptosis occurs in Hep3B cells at 72 h and 96 h after knocking down both HDAC1 and HDAC2. From the cell cycle analysis, the percentage of cells in the subG1 phase was plotted for the respective siRNA treatments in HEP3B cells, at 72 and 96 hours post-transfection. Data is representative of 3 independent experiments.

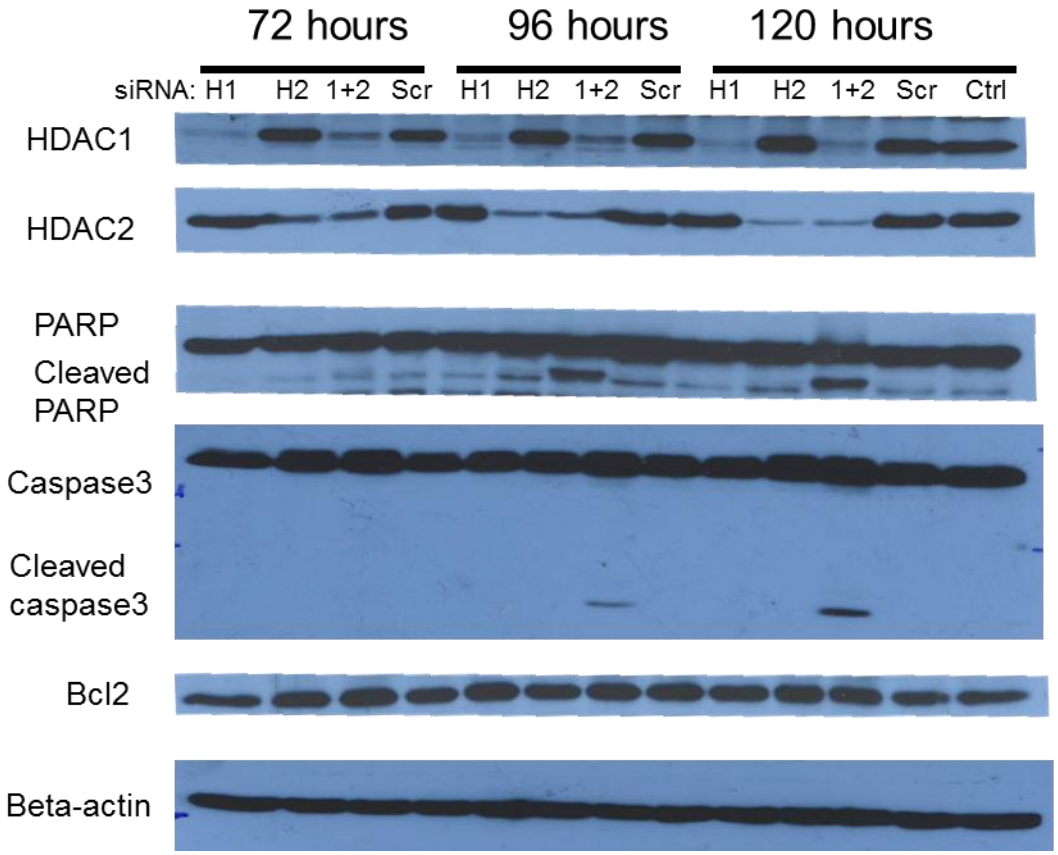


Figure 4.16 Increase in apoptotic proteins after HDAC1 and 2 knockdown. Expression of apoptotic proteins in HEP3B at various timepoints after being transfected with siRNA against HDAC1 (H1) or HDAC2 (H2) or both (1+2) or non-silencing control (Scr) or untransfected control (ctrl). There was cleavage of caspase 3 as well as PARP. However, the expression of Bcl2 remained unchanged. Beta-actin was used as the loading control.

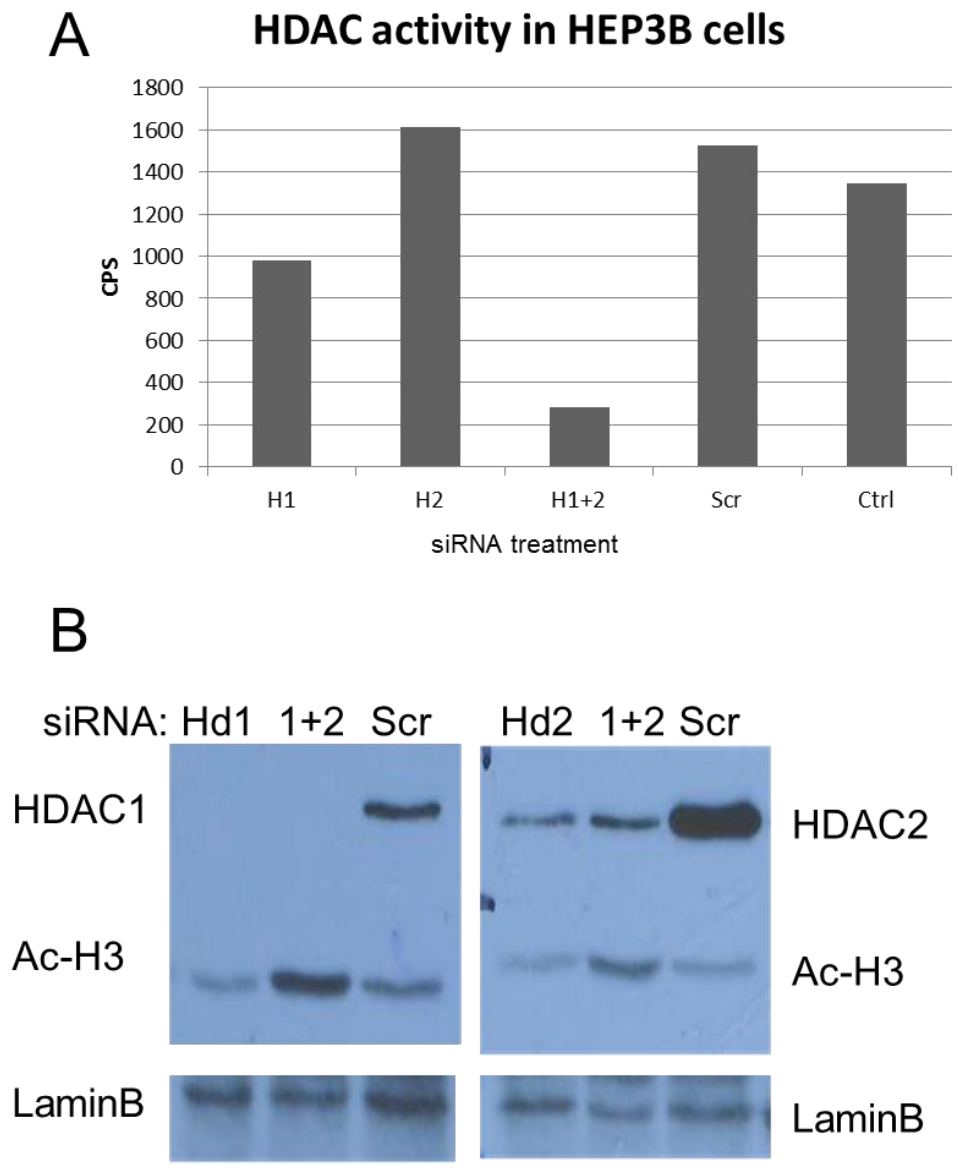


Figure 4.17 HDAC activity in HEP3B cells is synergistically reduced by HDAC1 and 2 knockdown. A, HEP3B cells were transfected with siRNA against HDAC1 (H1) or HDAC2 (H2) or both (1+2) or non-silencing control (Scr) or untransfected control (ctrl) for 48 hours before the nuclear extract was used to test for HDAC activity. Data is obtained from duplicates and is representative of 3 experiments. B, Western blot shows effective and specific knockdown of HDAC1 or/and HDAC2. Acetylated histone H3 (Ac-H3) was upregulated after knocking down both HDAC1 and 2. Lamin B was used as loading control for the nuclear extract.

silencing HDAC1 can partially reduce HDAC activity. When both HDAC1 and 2 were knocked down together, there was synergistic reduction in HDAC activity in the cells. This correlates with the colony formation assay results. Figure 4.17B shows that the nuclear extract used in the HDAC assay was verified to show effective HDAC1 or/and HDAC2 knockdown, as well as an upregulation of acetylated histone H3 protein (AcH3) after knocking down both HDAC1 and 2 together. This acetylation of histone H3 protein further demonstrated a reduction of global HDAC activity in the cell.

Similar to HEP3B cells, while knocking down HDAC2 has no effect on the global HDAC activity of HCT116 cells, knocking down HDAC1 can partially reduce HDAC activity (Figure 4.18). In the HCT116 p53-/- cells which do not produce functional HDAC2 protein, their basal HDAC activity is similar to that of HCT116 cells. However, when HDAC1 was knocked down in the HCT116 p53-/- cells, their HDAC activity level dropped dramatically.

4.4.2 Construction and verification of HDAC1 and HDAC2 wildtype and mutant expression plasmids

Both HDAC1 and 2 are enzymes that are known to deacetylate histone tails. To study the role of HDAC1 and HDAC2 in contributing to the HDAC activity in the cell, overexpression plasmids were constructed. Full length HDAC1 and HDAC2 were cloned into pcDNA3.1 vector. Site-directed mutagenesis was done to mutate 2 nucleotides within the catalytic domain of HDAC1 to generate an enzyme-dead mutant based on Hassig et al. (Hassig et al., 1998). To verify the constructed plasmids, we tried to overexpress them in HEP3B cells. While both the wildtype and mutant HDAC1 plasmid can be overexpressed, the HDAC2 plasmids failed to be overexpressed at the protein level (Figure 4.19A). Similar results was observed when

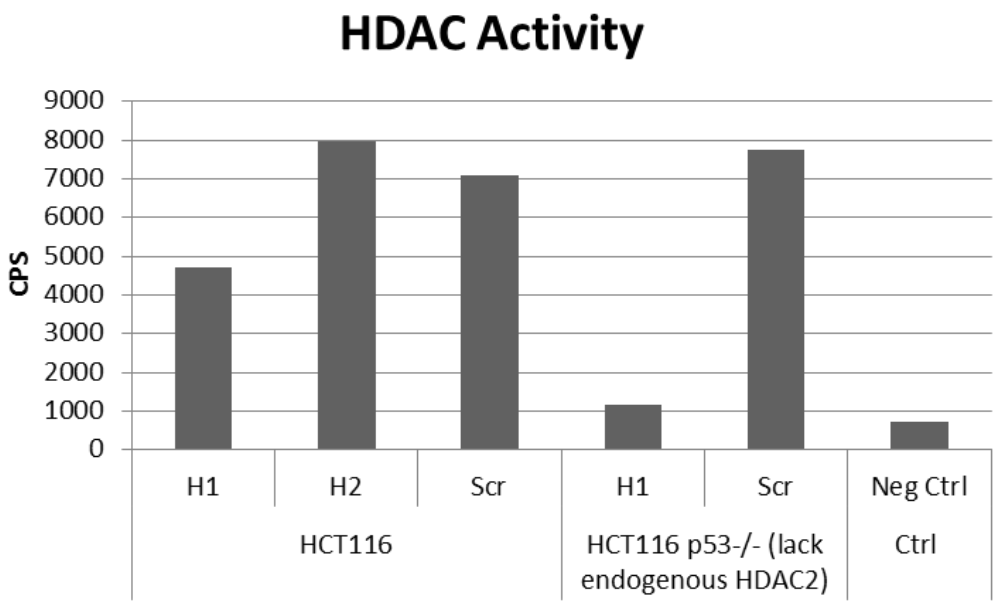


Figure 4.18 In HCT116 p53-/- cells which did not have endogenous HDAC2, knockdown of HDAC1 can dramatically reduce HDAC activity. HCT116 and HCT116 p53-/- cells were transfected with siRNA against HDAC1 (H1) or HDAC2 (H2) or non-silencing control (Scr) for 48 hours before the nuclear extract was used to test for HDAC activity. Negative control (Neg Ctrl) was performed by omitting the addition of the nuclear extract to the substrate in the assay. Knocking down HDAC1, but not HDAC2, can partially reduce HDAC activity in HCT116 cells. In HCT116 p53-/- cells which do not produce functional HDAC2 proteins, knocking down HDAC1 alone can markedly reduce HDAC activity level. Data is representative of 3 experiments.

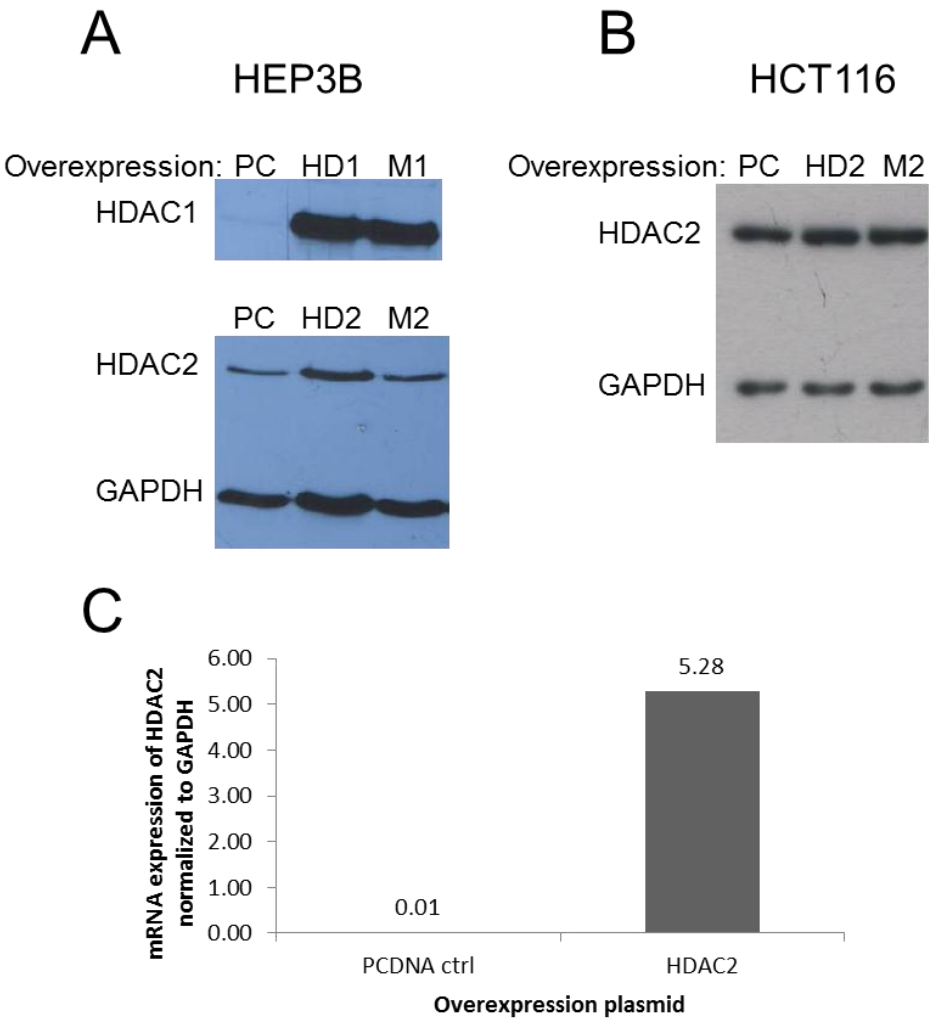


Figure 4.19 HDAC plasmid cannot be overexpressed at protein level. A, HEP3B cells were transfected with either pcDNA empty vector control (PC), HDAC1 wildtype plasmid (HD1), HDAC1 mutant plasmid (M1), HDAC2 wildtype plasmid (HD2), or HDAC2 mutant plasmid (M2) for 48 hours before protein extraction. Western blots showed the protein expression of HDAC1 and HDAC2 proteins. GAPDH was used as loading control. While HDAC1 wildtype and mutant plasmid can overexpress the protein, both the HDAC2 plasmids did not. B, Both the HDAC2 wildtype and mutant plasmids failed to be overexpressed in HCT116 cells. C, HCT116 cells were transfected with either HDAC2 overexpression vector (HDAC2) or pcDNA empty vector (pcDNA) for 48hours before RNA extraction, followed by real time RT-PCR using Roche Lightcycler. The HDAC2 mRNA was overexpressed over 500 folds.

we attempt to overexpress HDAC2 in HCT116 cells (Figure 4.19B), even though the plasmid was overexpressed at the mRNA level (Figure 4.19C). Therefore, we used the HCT116 p53-/- cell line which has a deletion on its HDAC2 DNA sequence due to microsatellite instability (Figure 4.20A). This led to a frameshift mutation and an early stop codon, resulting in a protein with only 30 amino acids instead of the full-length 488 amino acids, and cannot be detected on Western blot (Figure 4.20B). Without endogenous expression of HDAC2, both HDAC2 wildtype and mutant protein can be expressed in this HCT116 p53-/- cell line, along with HDAC1 (Figure 4.20C). While the HDAC1 plasmid of the same sequence has been previously verified by Hassig et al. (1998), the HDAC2 plasmid was not. After immunoprecipitating the wildtype and mutant HDAC2 plasmids using antibodies, we tested their HDAC activity after being overexpressed in the HCT116 p53-/- cells. Figure 4.21 confirmed that while the immunoprecipitated wildtype HDAC2 conferred HDAC activity, the mutant HDAC2 did not.

4.4.3 Effect of wildtype and mutant HDAC1 plasmid on rescuing effect of HDAC1 and 2 knockdown

To find out if the reduction of colony formation due to HDAC1 and 2 knockdown can be rescued by the wildtype or enzyme-dead mutants of HDAC1 and 2, a rescue experiment was performed. HEP3B cells were transfected with either the wildtype HDAC1+2 plasmid, mutant HDAC1+2 plasmid, or empty pcDNA vector before being transfected with siRNA against HDAC1+2 or non-silencing control. This was done in this order so that the overexpression plasmids had time to produce the HDAC proteins before the siRNA would prevent their expression. However, only HDAC1, but not HDAC2, protein expression was rescued partially (Figure 4.22A). This was consistent with previous experiment where HDAC2 protein cannot be

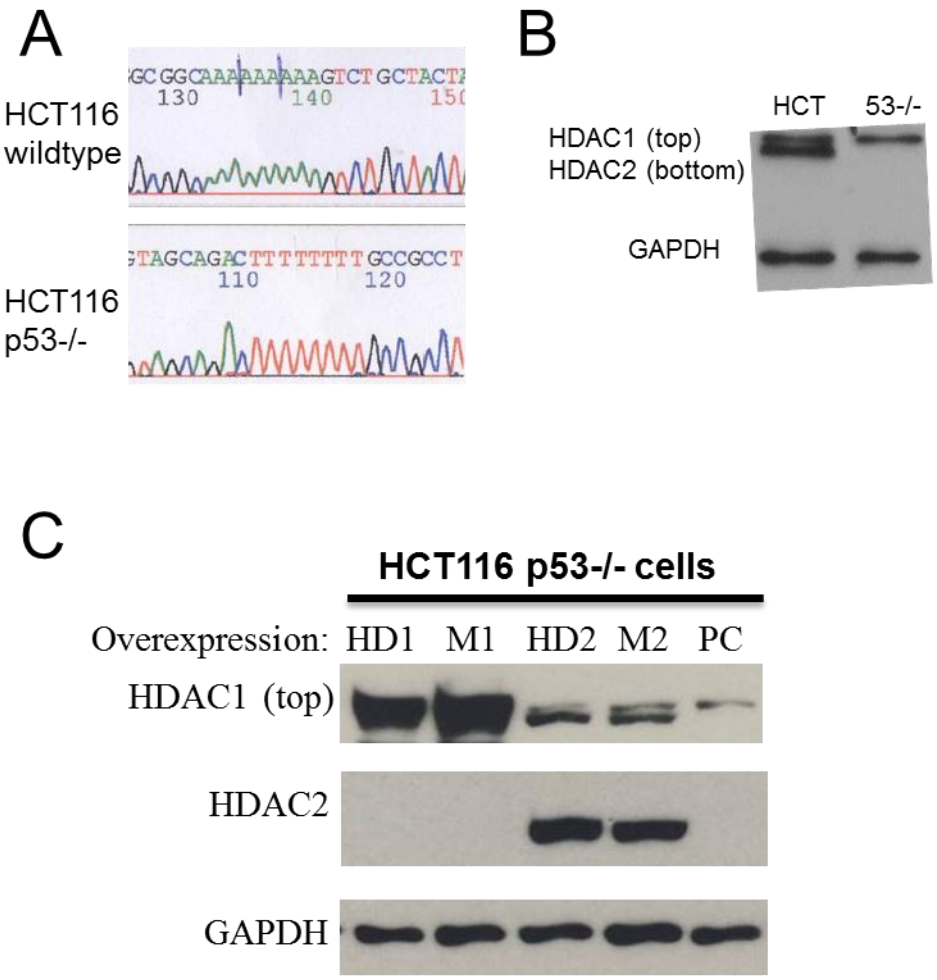


Figure 4.20 Both HDAC1 and HDAC2 wildtype and mutant plasmids can be overexpressed in HCT116 p53-/- cells. A, HCT116 p53-/- cells contained a single nucleotide deletion which resulted in a frameshift mutation. Sequencing of the cDNA from HCT116 wildtype cells showed 9 adenosine/thymidine residues while that from the same region of the HCT116 p53-/- cells showed only 8 adenosine/thymidine residues. B, Unlike HCT116 cells, the HCT116 p53-/- cells do not have endogenous full-length HDAC2 protein. C , HCT116 p53-/- cells were transfected with either pcDNA empty vector control (PC), HDAC1 wildtype plasmid (HD1), HDAC1 mutant plasmid (M1), HDAC2 wildtype plasmid (HD2), or HDAC2 mutant plasmid (M2) for 48 hours before protein extraction. Western blots showed the protein expression of HDAC1 and HDAC2 proteins. GAPDH was used as loading control

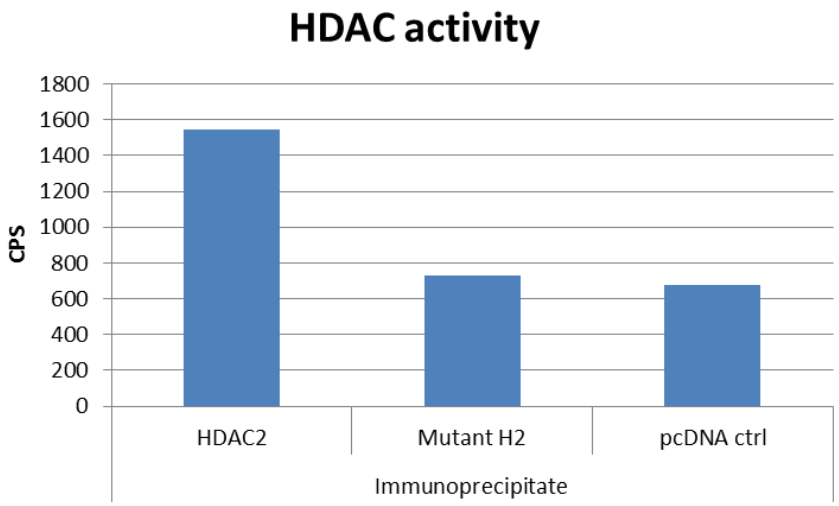


Figure 4.21 HDAC activity after overexpression of HDAC2 wildtype and mutant plasmids in HCT116 p53-/- cells which lack endogenous full-length HDAC2. Cells were transfected with either wildtype HDAC2 (HDAC2) or mutant HDAC2 (Mutant H2) or empty vector control (pcDNA ctrl) for 48 hours before the nuclear extract was immunoprecipitated with anti-HDAC2 antibodies and tested for HDAC activity. The wildtype HDAC2 plasmid was verified to confer HDAC activity while the mutant HDAC2 plasmid did not.

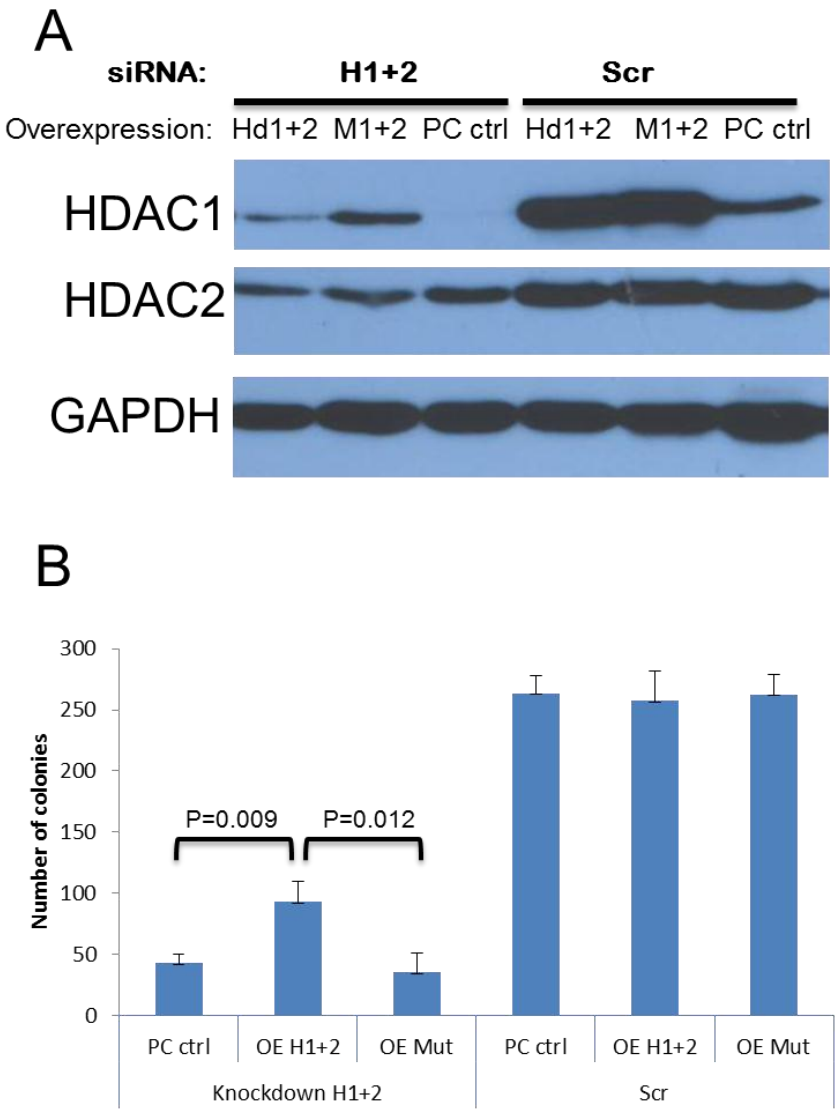


Figure 4.22. Rescue experiment. HEP3B cells were transfected with either wildtype HDAC1 and 2 plasmids (Hd1+2), or mutant HDAC1 and 2 plasmids (M1+2), or empty vector pcDNA (PC ctrl). They were allowed to recover overnight before being transfected with either HDAC1 and 2 siRNA (H1+2) or non-silencing control (Scr). The cells were allowed to recover overnight before being replated for colony formation. A, Western blot shows the overexpression plasmids can rescue the expression of HDAC1 but not that of HDAC2. GAPDH was used as loading control. B, The overexpression of the wildtype plasmids, but not the mutant, can partially rescue the reduction of colony formation after knocking down HDAC1 and 2. Error bars showed standard deviations.

overexpressed except in a cell line that did not produce endogenous full-length HDAC2. Nevertheless, the partial rescue in HDAC1 expression was able to partially rescue the reduction of colony formation due to knockdown of both HDAC1 and 2 (Figure 4.22B). This effect was only observed when the wildtype plasmids, but not the mutant, were used. HDAC activity assay showed that the HDAC activity in the cell was increased only when the wildtype but not the mutant plasmid was used in the rescue (Figure 4.23), attributing the partial rescue effect in colony formation to the enzymatic HDAC activity.

4.4.4 Protective effects of wildtype HDAC1 against PXD101-induced cell death

Using the HCT116 p53-/- cell line which lacks endogenous full length HDAC2, we were able to overexpress both the HDAC1 and 2 wildtype of mutant plasmids. Using this cell line, we examined the protective effect of HDAC1 and 2 wildtype and mutant plasmids on cell death induced by HDAC inhibitor PXD101. Figure 4.24 showed the dose response of PXD101-induced cell death in the HCT116 p53-/- cells. Increasing the dosage of PXD101 increased the percentage of cells undergoing apoptosis. Figure 4.25 showed that the overexpression of wildtype HDAC1 alone can partially protect the cells from PXD101-induced cell death. Overexpression of both HDAC1 and HDAC2 together did not increase the protective effect. The mutant HDAC1 failed to exhibit the same protective effect as the wildtype. Figure 4.26 showed that overexpression of the wildtype HDAC1, either alone ($p= 0.005$) or together with wildtype HDAC2 ($p=0.004$), can increase global HDAC activity of the cells. However, the wildtype HDAC2 plasmid alone ($p=0.067$) did not significantly increase global HDAC activity. Also, neither the mutant HDAC1 nor mutant HDAC2 can increase HDAC activity. In fact, mutant HDAC1 actually reduced HDAC activity ($p=0.028$) possibly by competing with the endogenous

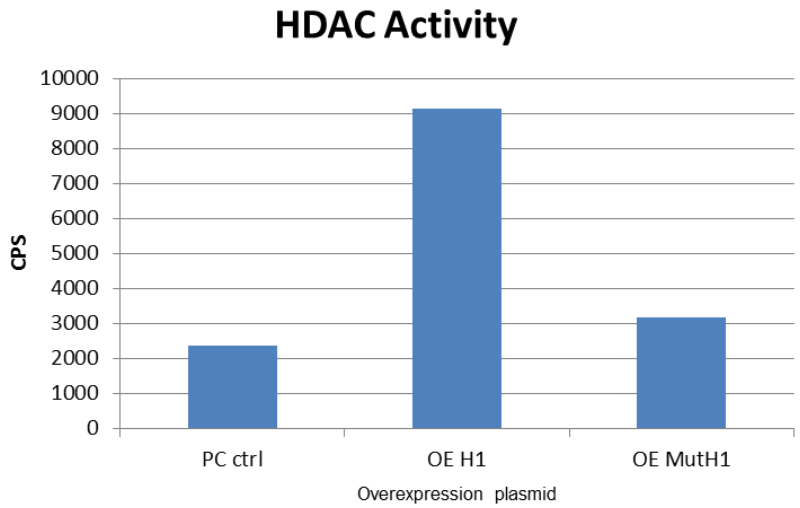


Figure 4.23 Rescue of HDAC activity. HEP3B cells were transfected with either wildtype HDAC1 and 2 plasmids (Hd1+2), or mutant HDAC1 and 2 plasmids (M1+2), or empty vector pcDNA (PC ctrl). They were allowed to recover overnight before being transfected with HDAC1 and 2 siRNAs. The nuclear extract was used to test for HDAC activity. The wildtype but not the mutant plasmids can rescue HDAC activity in these cells. It must be noted that only the expression of HDAC1, but not HDAC2 is rescued.

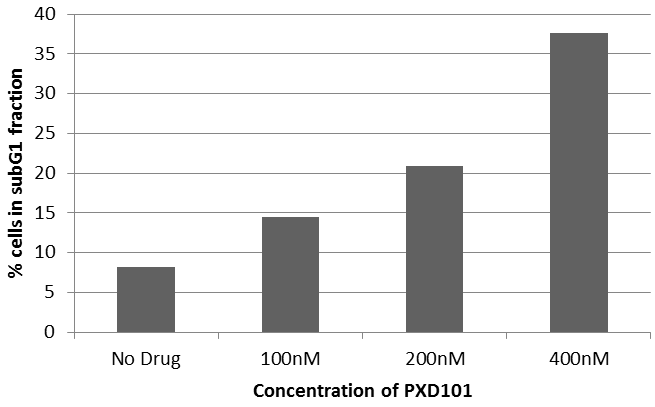


Figure 4.24 Dose response of PXD101-induced apoptosis in HCT116 p53-/- cells. Cells were treated with 100nM, 200nM, or 400nM PXD101 or vehicle control for 48 hours. The subG1 fraction of cells, indicative of apoptotic cells, increased after treatment with increasing doses of the HDAC inhibitor PXD101.

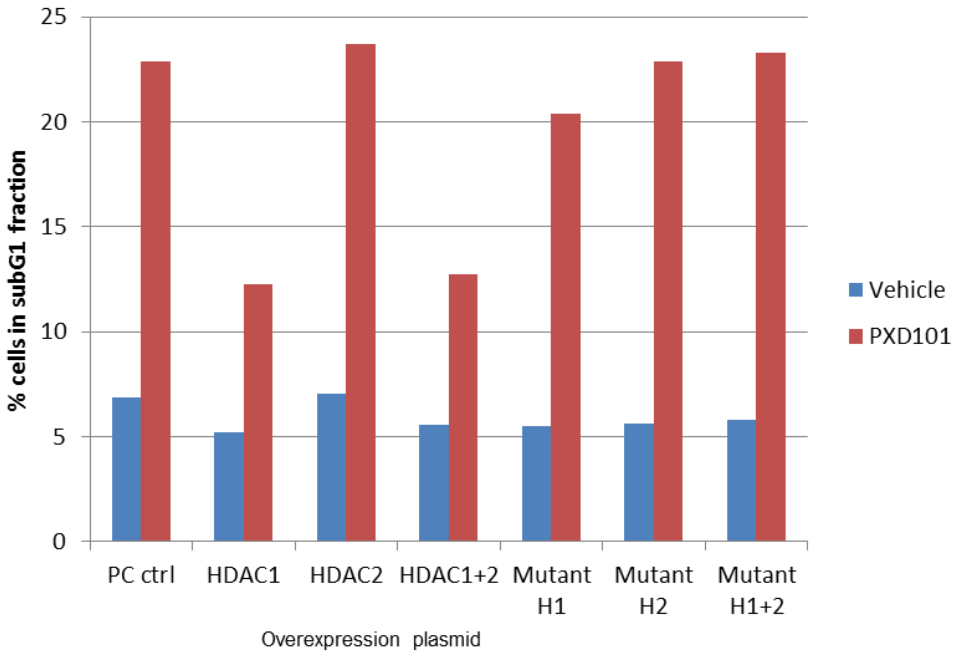


Figure 4.25 Protective effect of HDAC1 overexpression against PXD-induced death in HCT116 p53-/- cells. Cells were transfected with the respective plasmids for 24 hours before they were treated with 300nM PXD101 or vehicle control for 48 hours. The subG1 fraction of cells, indicative of apoptotic cells, increased after treatment with PXD101. Overexpression of wildtype HDAC1, but not HDAC2, can partially rescue cells from PXD101 induced cell death. This protective effect was not observed when mutant HDAC1 was overexpressed.

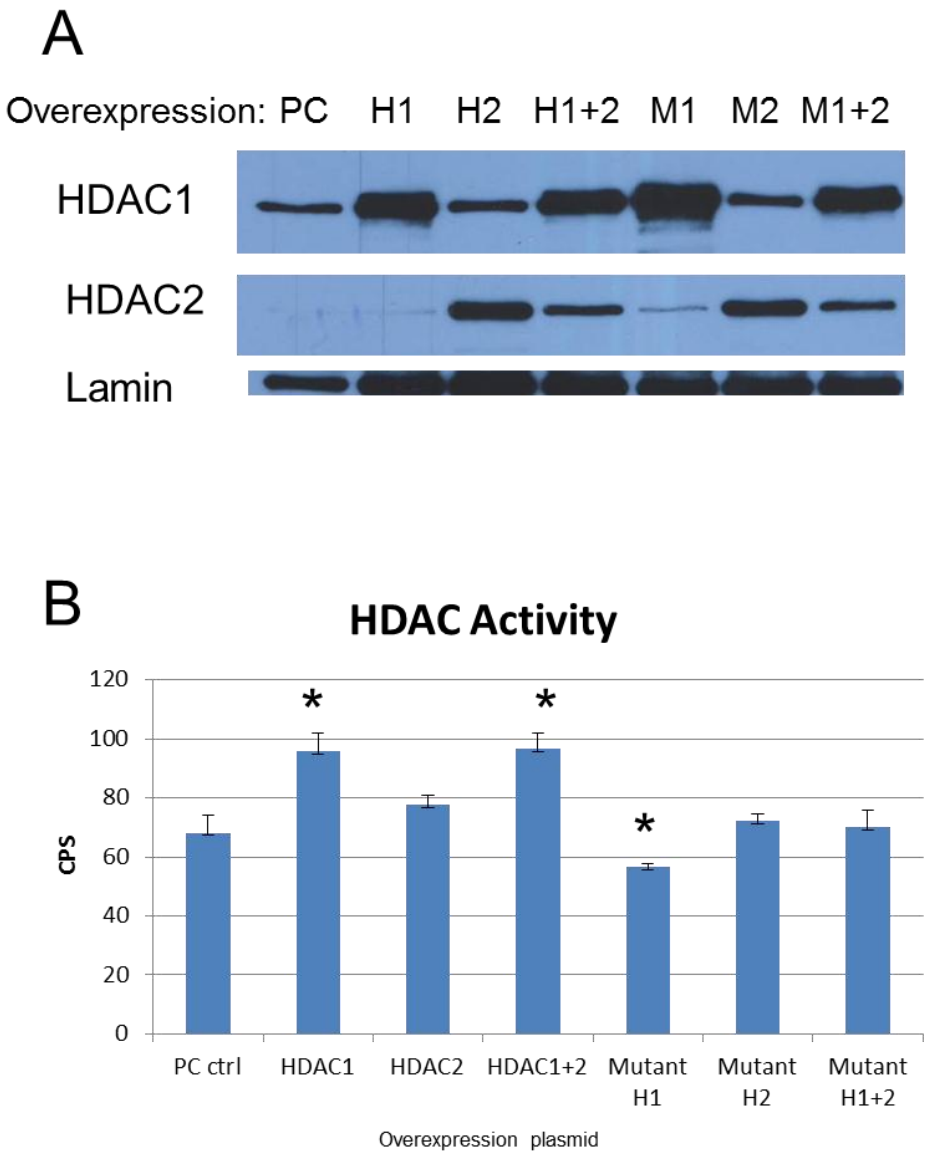


Figure 4.26 Effect of overexpressing wildtype and mutant HDAC1 and 2 on global HDAC activity in HCT116 p53-/- cells. Cells were transfected with the respective plasmids for 48 hours before they were harvested for extraction of nuclear protein. A, Western blot showed the overexpression of the respective proteins. B, HDAC activity assay showed that the overexpression of HDAC1, but not HDAC2 alone, can increase global HDAC activity in the cells compare to the empty vector control (PC ctrl). Neither the mutant HDAC1 nor mutant HDAC2 can increase the global HDAC activity. Error bars showed standard deviations. The * indicates $p<0.05$ when compared to the empty vector PC control.

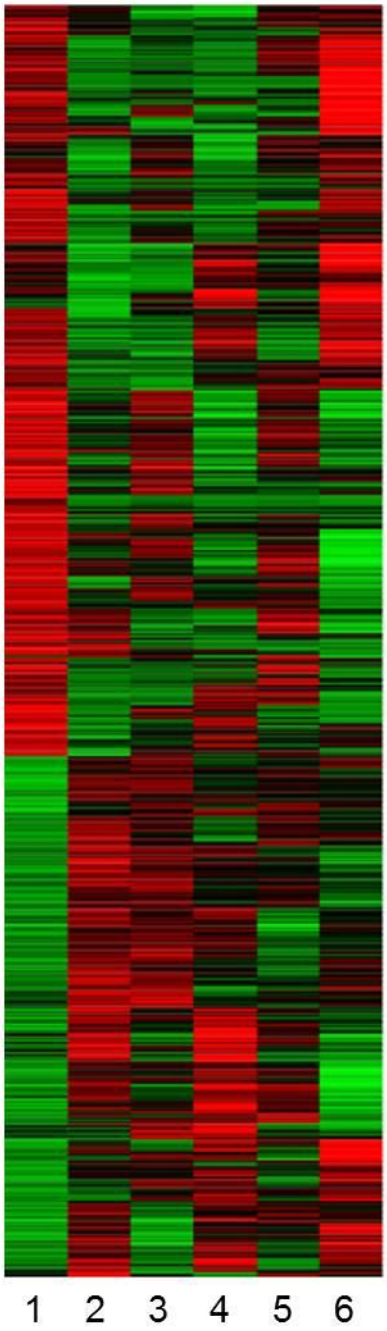
HDAC1. Together, these results demonstrated that restoring HDAC activity by overexpression of wildtype HDAC1 alone can partially protect the cells against drug-induced cell death.

4.5 Gene expression profiles of Hep3B cells after knockdown of HDAC1 or/and HDAC2 and PXD101 treatment

4.5.1 Microarray analysis

Microarray was used to analyze the gene expression profiles of HEP3B cells after knocking down HDAC1, HDAC2, or both. An HDAC inhibitor PXD101 was also used for comparison with the HDAC1 and 2 specific knockdown. Figure 4.27 shows the number of genes upregulated and downregulated by each treatment and a heat map to illustrate the results. In the Illumina HumanRef-8 Beadchip which contained 24,526 coding transcripts in total, there were 2057 genes that were regulated at least 2 folds by the treatments. Of these, 409 genes were differentially regulated by silencing both HDAC1 and HDAC2 together but not individually. Classification of the differentially regulated genes were done based on Gene Ontogeny to show the molecular functions, biological processes and cellular components to which these genes belong (Figure 4.28). Using the Protein ANalysis THrough Evolutionary Relationships (PANTHER) pathway database, these genes were also grouped into the pathways with which they are associated (Thomas et al., 2003). Table 4.4 shows the list of pathways. Of the 146 pathways that were hit, the ones which contained at least 5 genes that are regulated by knocking down both HDAC1 and 2 together were highlighted in bold. These are the angiogenesis pathway, the heterotrimeric G protein signalling pathway (G1 alpha and Gs alpha mediated), inflammation pathway mediated by chemokines and cytokines, the integrin signalling

A



B

	UP	DOWN
HDAC1	885	1168
HDAC2	758	1299
HDAC1+2	997	1048
PXD	1208	836

Figure 4.27 Microarray analysis to study effect of HDAC1 or/and HDAC2 knockdown on gene expression in HEP3B cell. A, Heat map to show gene expression in HEP3B cells after knocking down HDAC1 or/and 2 with siRNAor treatment with PXD101. Red indicates upregulation and green indicates downregulation. 1, Non-silencing control SCR. 2, HDAC1 siRNA. 3, HDAC2 siRNA. 4, HDAC1 and 2 siRNA. 5, Untransfected vehicle control. 6, PXD101 treatment. A total of 2057 genes that were regulated by at least 2 folds compared to the controls after siRNA or PXD101 treatment were clustered. B, Table to show the number of genes upregulated and downregulated after each treatment.

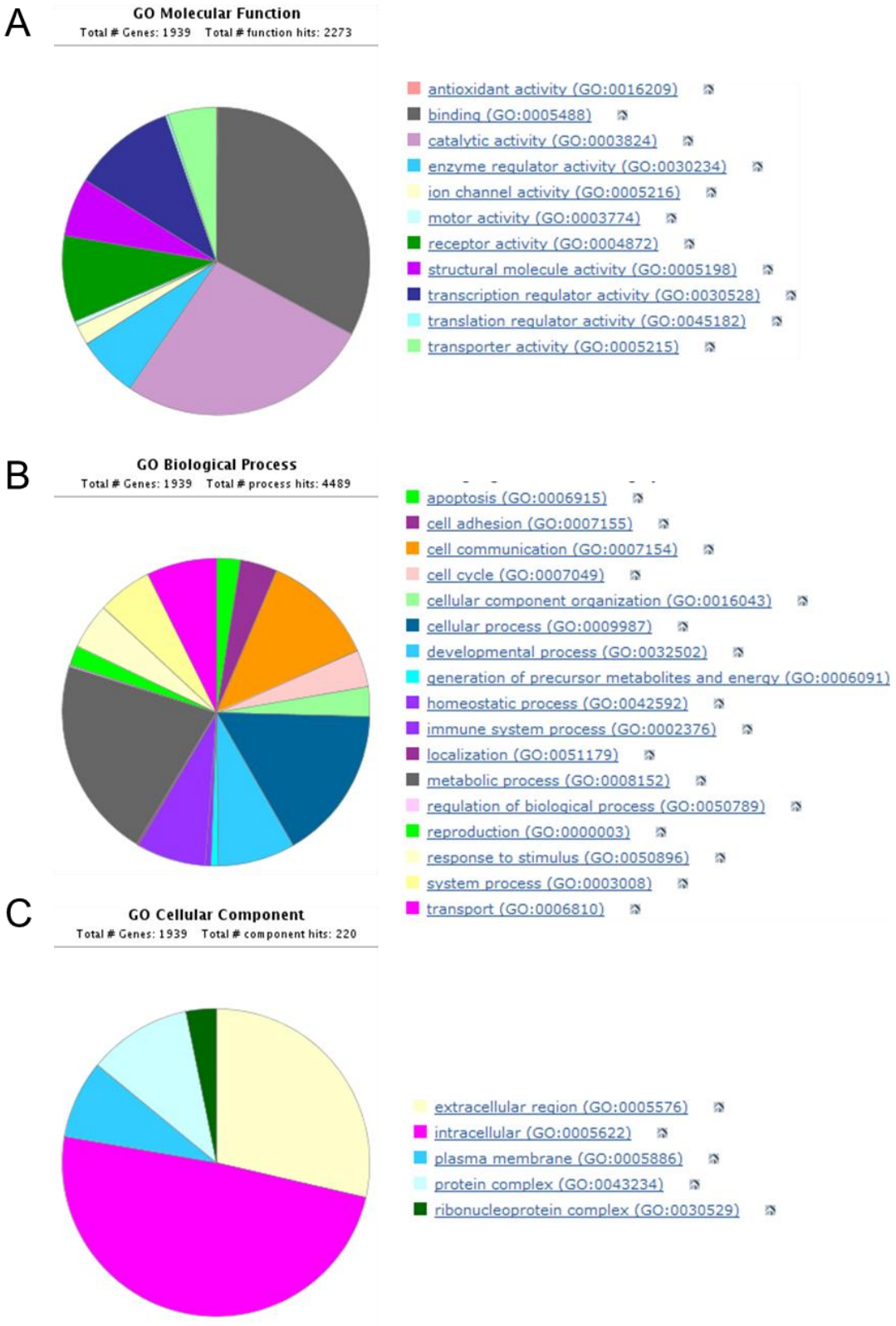


Figure 4.28 Pie chart to show genes that were regulated at least 2 fold compared to the control after siRNA or PXD101 treatment in Hep3B cells. The classification of genes into molecular function (A), biological processes (B), and cellular component (C) was based on gene ontogeny.

Table 4.4. List of pathways with genes regulated by knocking down both HDAC1 and 2 together but not individually. The pathways with 5 or more genes being regulated at least 2 folds after knocking down both HDAC1 and 2 together is in bold.

5HT1 type receptor mediated signaling pathway (P04373)	Huntington disease (P00029)
5HT2 type receptor mediated signaling pathway (P04374)	**Inflammation mediated by chemokine and cytokine signaling pathway**
5HT4 type receptor mediated signaling pathway (P04376)	**Integrin signalling pathway (P00034)**
Alpha adrenergic receptor signaling pathway (P00002)	Interleukin signaling pathway (P00036)
Alzheimer disease-amyloid secretase pathway (P00003)	JAK/STAT signaling pathway (P00038)
Alzheimer disease-presenilin pathway (P00004)	Mannose metabolism (P02752)
Angiogenesis (P00005)	Metabotropic glutamate receptor group I pathway (P00041)
Apoptosis signaling pathway (P00006)	Metabotropic glutamate receptor group II pathway (P00040)
Asparagine and aspartate biosynthesis (P02730)	Metabotropic glutamate receptor group III pathway (P00039)
Axon guidance mediated by Slit/Robo (P00008)	Muscarinic acetylcholine receptor 1 and 3 signaling pathway
Axon guidance mediated by semaphorins (P00007)	Muscarinic acetylcholine receptor 2 and 4 signaling pathway
B cell activation (P00010)	Nicotinic acetylcholine receptor signaling pathway (P00044)
Beta1 adrenergic receptor signaling pathway (P04377)	Notch signaling pathway (P00045)
Beta2 adrenergic receptor signaling pathway (P04378)	Oxidative stress response (P00046)
Beta3 adrenergic receptor signaling pathway (P04379)	Oxytocin receptor mediated signaling pathway (P04391)
Blood coagulation (P00011)	**PDGF signaling pathway (P00047)**
Cadherin signaling pathway (P00012)	PI3 kinase pathway (P00048)
Cell cycle (P00013)	Parkinson disease (P00049)
Cortocotropin releasing factor receptor signaling pathway	Pentose phosphate pathway (P02762)
Cytoskeletal regulation by Rho GTPase (P00016)	Plasminogen activating cascade (P00050)
EGF receptor signaling pathway (P00018)	Pyrimidine Metabolism (P02771)
Endogenous_cannabinoid_signaling (P05730)	Ras Pathway (P04393)
Endothelin signaling pathway (P00019)	Salvage pyrimidine deoxyribonucleotides (P02774)
FAS signaling pathway (P00020)	Salvage pyrimidine ribonucleotides (P02775)
FGF signaling pathway (P00021)	Synaptic_vesicle_trafficking (P05734)
Fructose galactose metabolism (P02744)	T cell activation (P00053)
GABA-B_receptor_II_signaling (P05731)	TCA cycle (P00051)
General transcription regulation (P00023)	TGF-beta signaling pathway (P00052)
Glycolysis (P00024)	Thyrotropin-releasing hormone receptor signaling pathway
Hedgehog signaling pathway (P00025)	Toll receptor signaling pathway (P00054)
Heterotrimeric G-protein signaling pathway-Gi α & Gs α mediated pathway	Transcription regulation by bZIP transcription factor
Heterotrimeric G-protein signaling pathway-Gq α & Go α mediated pathway	VEGF signaling pathway (P00056)
Heterotrimeric G-protein signaling pathway-rod outer segment phototransduction	Vitamin D metabolism and pathway (P04396)
Histamine H1 receptor mediated signaling pathway	**Wnt signaling pathway (P00057)**
Histamine H2 receptor mediated signaling pathway	p53 pathway feedback loops 2 (P04398)
	p53 pathway (P00059)

pathway, the platelet-derived growth factor (PDGF) signalling pathway, and the Wnt signalling pathway.

4.5.2 Quantitative RT-PCR to validate selected genes

To investigate the genes that mediate the effects of HDAC1 and 2 knockdown on reduced colony formation and increased apoptosis, some candidate were selected for validation by RT-PCR. These genes were selected if they were regulated significantly by HDAC inhibitor PXD101 treatment and by knocking down both HDAC1 and 2 but not individually. Genes that have known some functions in cell survival and proliferation were shortlisted. Table 4.5 shows the list of 9 genes selected, with their fold changes after each treatment and their functions. The genes that were upregulated after silencing both HDAC1 and 2 were CYGB, DYRK4, GALR2, GLUT3, MAGEC2, NMES1, and NOTCH3; the downregulated genes were LOX and LOXL4. Figure 4.29 shows the quantitative RT-PCR results of these genes. Their relative mRNA expression was consistent with the microarray data.

4.5.3 Western Blot to validate gene candidates

After validating the RNA expression of the candidate genes, Western blot was done to check the protein expression of some of these candidate genes after silencing HDAC1 or/and 2. Figure 4.30 showed that among the upregulated genes after HDAC1+2 knockdown, only CYGB, GALR2 and PLCg2 demonstrated upregulation at the protein level, while MAGEC2 and NMES did not. On the other hand, both of the downregulated genes selected (LOX and LOXL4) showed downregulation at protein level consistent with the microarray data.

4.5.4 Effect of HDAC-regulated genes LOX and LOXL4 on colony formation in HEP3B cells

Table 4.5. Functions and fold change of genes selected for RT-PCR validation. The * indicates the gene s downregulated while the rest were upregulated after HDAC1+2 knockdown.

Gene	Fold change after treatment				Functions
	H1	H2	H1+2	PXD	
CYGB	1.03	1.21	4.30	0.82	ubiquitously expressed hexacoordinate hemoglobin that may facilitate diffusion of oxygen through tissues, scavenge nitric oxide or other reactive oxygen species, or serve a protective function during oxidative stress; downregulation of cytoglobin as a key event in a familial cancer syndrome of the upper aerodigestive tract.
DYRK4	0.14	1.60	2.69	11.21	kinase; involved in cell proliferation, survival, and development
GALR2	2.98	1.15	5.68	3.48	decrease proliferation and increase apoptosis
GLUT3	1.66	1.39	4.17	56.45	potential prognostic marker for oral squamous cell carcinoma, interact with cancer and stem cell marker podocalyxin in malignant stem cell
MAGEC2	0.52	0.21	9.64	10.64	member of the MAGEC gene family; not expressed in normal tissues, except for testis, and is expressed in tumors of various histological types
NMES1	1.41	1.20	4.18	4.33	low expression in oesophageal squamous cell carcinoma; epigenetically regulated; inhibit cell motility in ESCC cells
NOTCH3	0.63	1.03	6.05	14.04	short cervical cancer patient survival with high Notch3; inactivation increase apoptosis
*LOX	0.45	0.46	0.04	0.11	negatively regulated by LKB1 through mTOR-HIF-1α signaling axis, mediates lung cancer progression. Inhibition of LOX activity dramatically alleviates lung cancer malignancy progression. Up-regulated LOX expression triggers excess collagen deposition in Lkb1-deficient lung tumors, and thereafter results in enhanced cancer cell proliferation and invasiveness through activation of β1 integrin signaling. High LOX level and activity correlate with poor prognosis and metastasis.
*LOXL4	0.52	0.51	0.14	0.23	overexpression of the LOXL4 mRNA and protein and a close relation of LOXL4 with the pathogenesis of head and neck squamous cell carcinomas

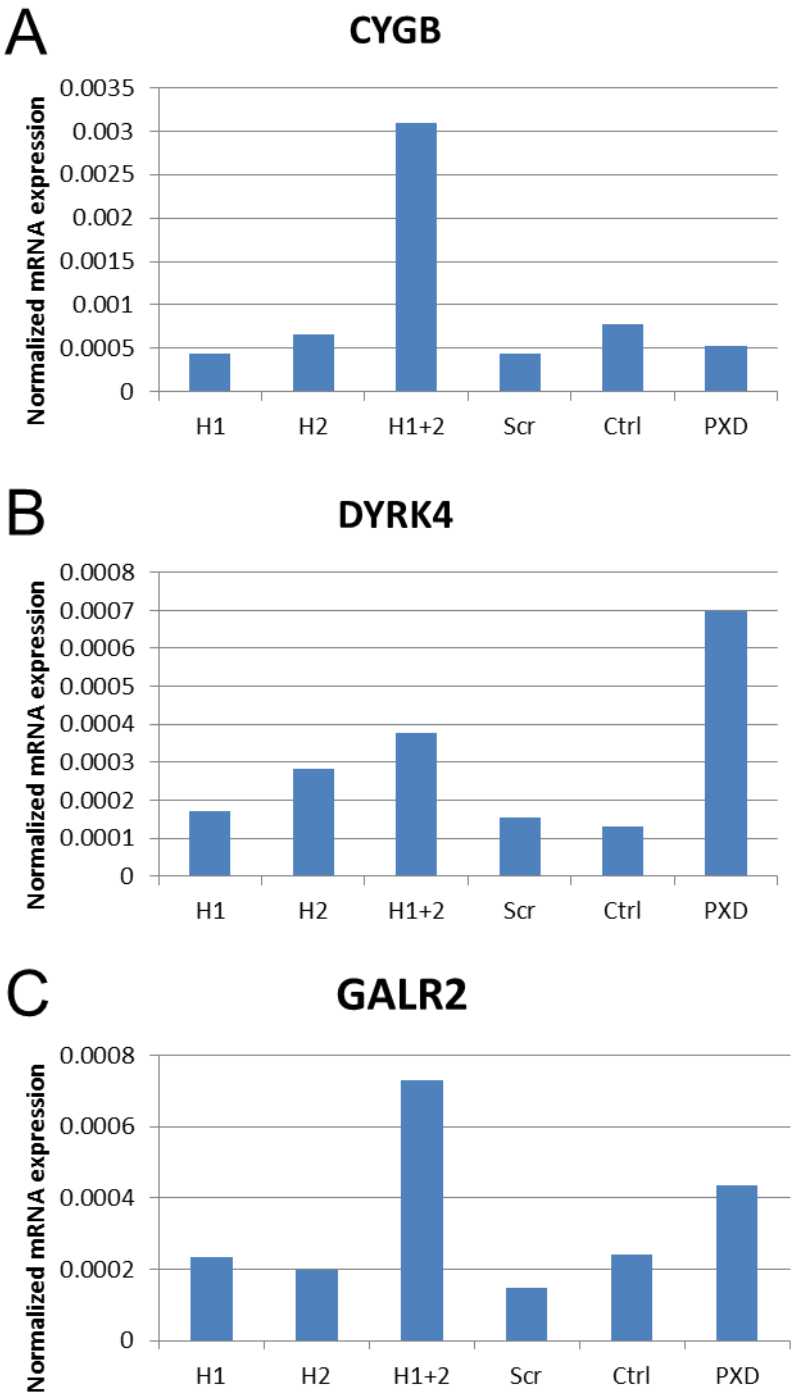


Figure 4.29 Validation of gene expression by quantitative real time RT-PCR. HEP3B cells were transfected with siRNA against HDAC1 (H1), or HDAC2 (H2), or both (H1+2), or non-silencing control (Scr) for 72 hours, or treated with 2000nM PXD101 (PXD) for 24 hours. RNA was extracted and used in real time quantitative RT-PCR using primers for the respective genes. Each sample was done in duplicates and the average was used to calculate the mRNA expression normalized against GAPDH.

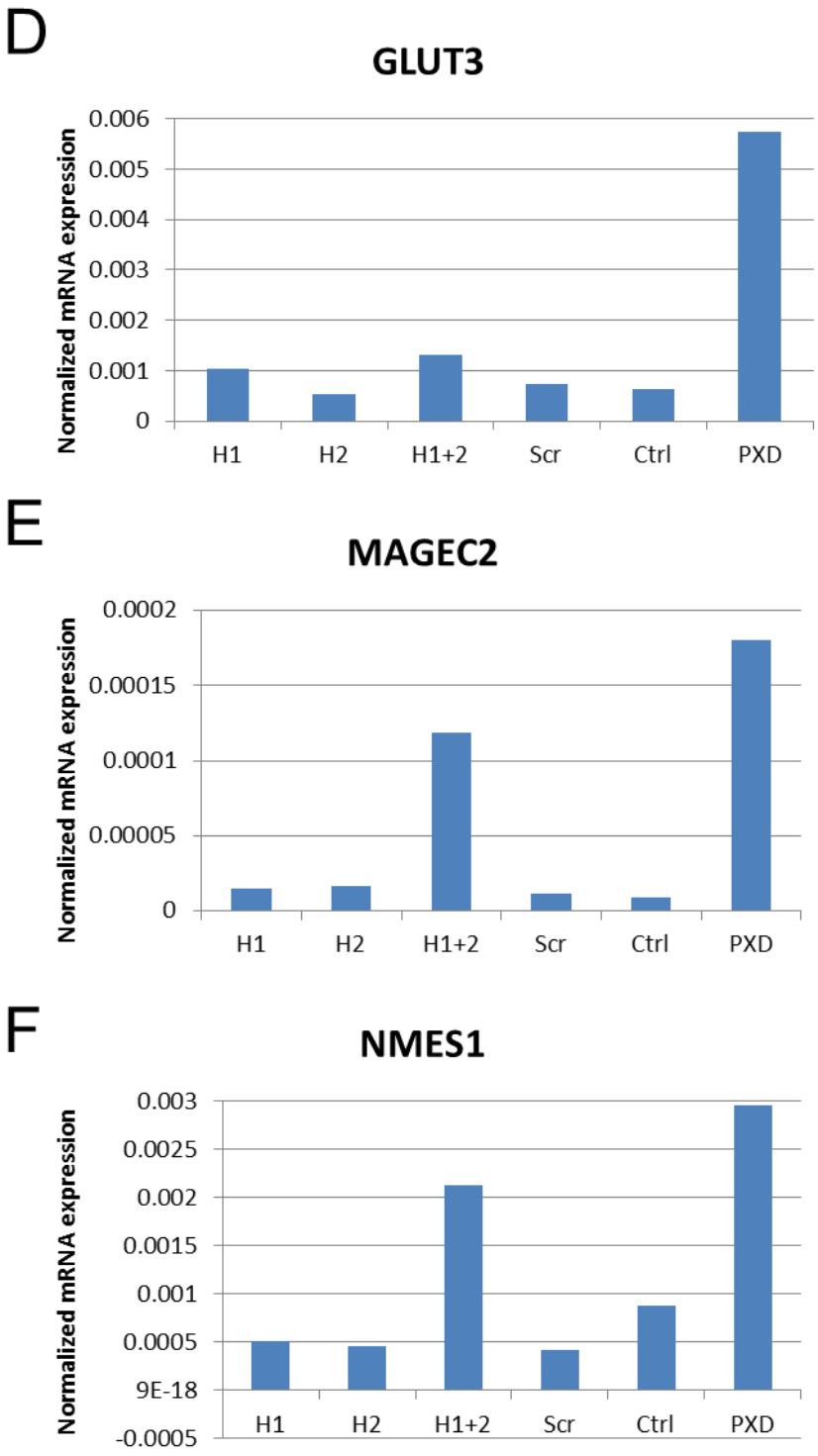


Figure 4.29 Validation of gene expression by quantitative real time RT-PCR.

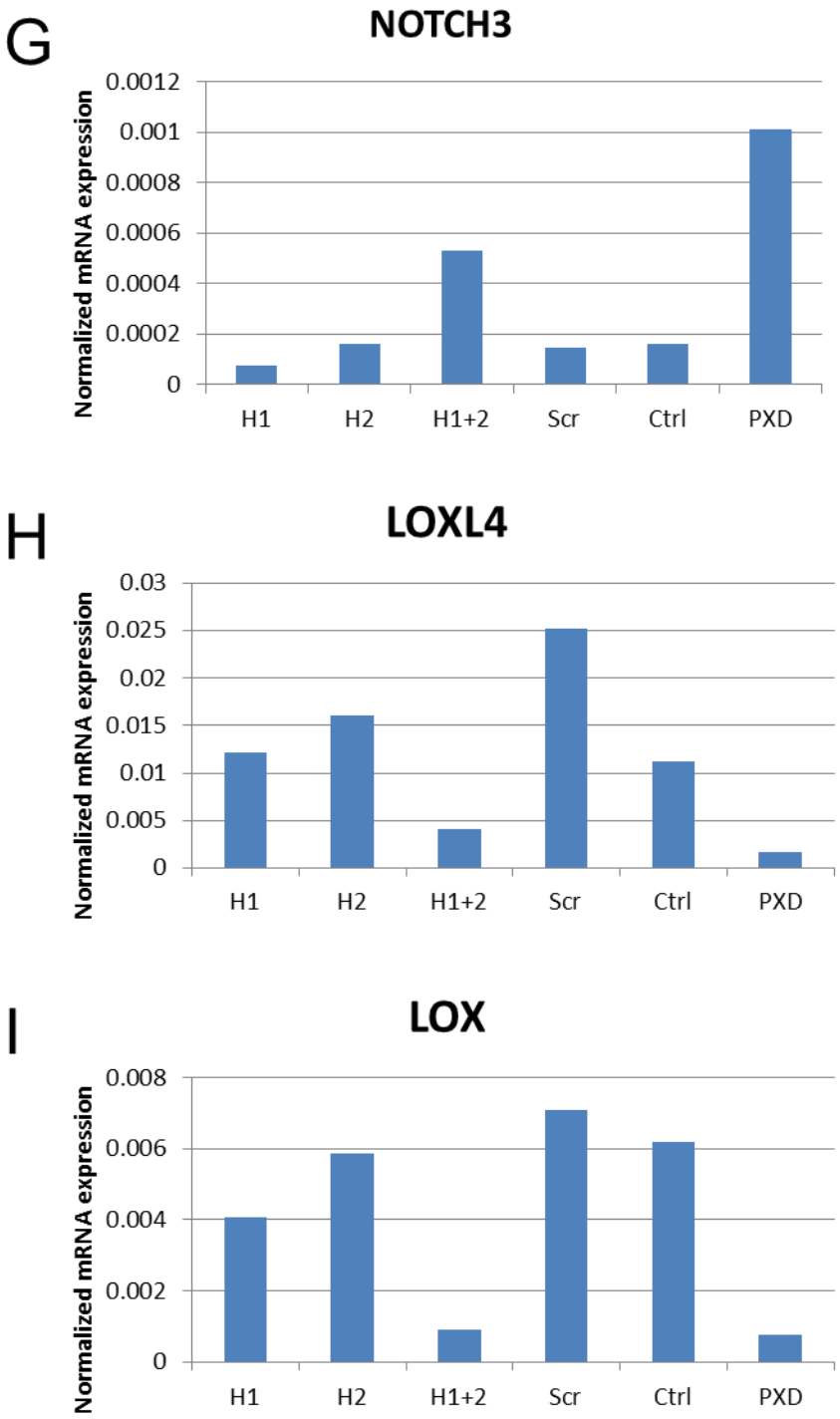


Figure 4.29 Validation of gene expression by quantitative real time RT-PCR.

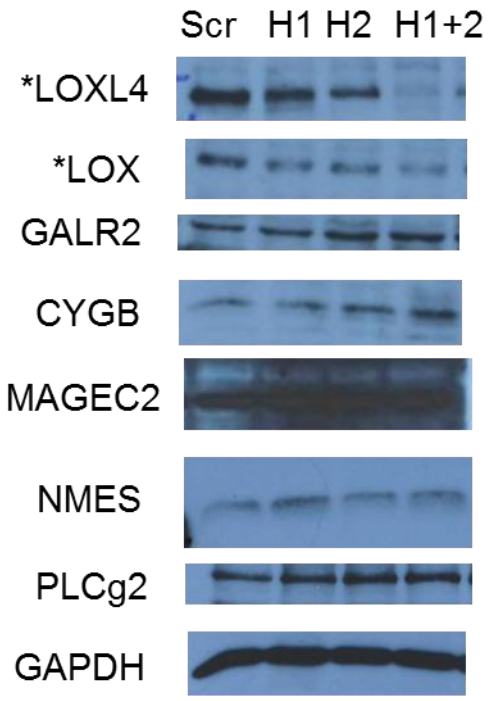


Figure 4.30 Validation of gene expression by Western blot. HEP3B cells were transfected with siRNA against either HDAC1 (H1) or HDAC2 (H2) or both (H1+2) or non-silencing control (Scr) for 72 hours. Cells were harvested and total protein extracted and quantified. Western blot was done using antibody against the respective proteins shown above. The * indicates the gene s downregulated while the rest were upregulated after HDAC1+2 knockdown based on microarray data. GAPDH was used as loading control.

There were 2 downregulated genes selected to test if they could be possible mediators of HDAC1+2 knockdown-induced cell death: LOX and LOXL4. Both of them belong to the LOX family of enzymes. From the microarray, LOX was found to be downregulated 24.4 fold by HDAC1+2 knockdown, and 8.8 fold by PXD101 treatment. LOXL4 was found to be downregulated 7.1 fold by HDAC1+2 knockdown, and 4.3 fold by PXD101 treatment.

The expression of LOX and LOXL4 were silenced using siRNA in HEP3B cells. Figure 4.31 shows that the siRNA against LOX and LOXL4 can efficiently knockdown specific genes. However, the knockdown of LOX or LOXL4 did not change colony formation in the HEP3B cells (Figure 4.32). In fact, the knockdown of both LOX and LOXL4 together increased the colony formation in these cells ($p=0.025$).

4.5.5 Effect of HDAC-regulated gene GALR2 on colony formation in HEP3B cells

The GALR2 gene was selected to test if its upregulation is a possible mediator of HDAC1+2 knockdown-induced cell death. From the microarray, GALR2 was found to be upregulated 5.7 fold by HDAC1+2 knockdown, and 3.5 fold by PXD101 treatment. The pCMV-GALR2 overexpression plasmid was requested from Dr Kanazawa from Jichi Medical University in Japan. Figure 4.33A shows that the pCMV-GALR2 plasmid can overexpress the gene in the HEP3B cells. The GALR2 overexpression slightly reduced colony formation in the HEP3B cells (Figure 4.33B), but it was not statistically significant ($p=0.11$).

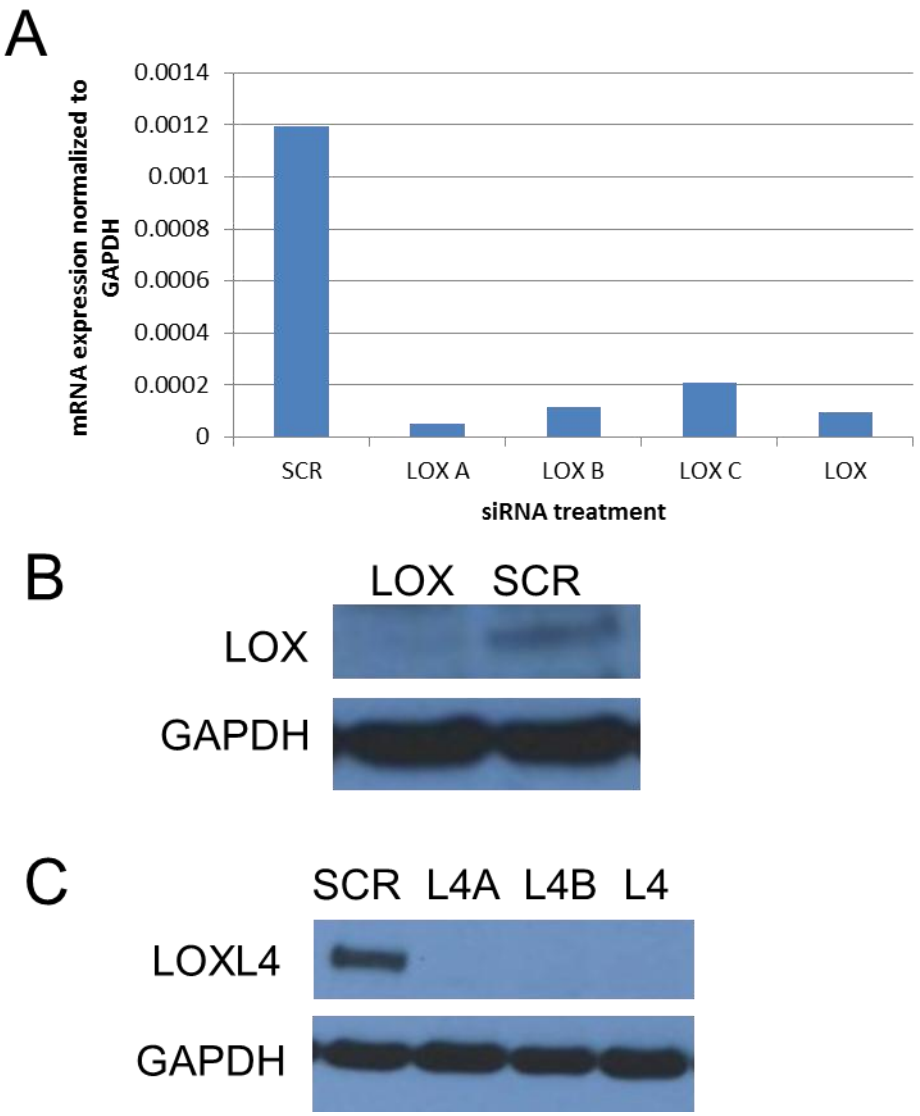


Figure 4.31 RT-PCR and Western blot to show efficiency of LOX and LOXL4 knockdown. A, HEP3B cells were transfected with either LOX siRNA (3 sequences) for 72 hours. Cells were harvested and RNA extracted. Real time quantitative RT-PCT was done to shown the LOX mRNA expression after each siRNA treatment (LOXA, B, or C), as well as when the 3 sequences of siRNA were combined together (LOXABC). All 3 siRNA sequences were able to knockdown LOX expression effectively so they were pooled together for use in subsequent experiments. B, The pooled LOX siRNA was able to knock down LOX protein expression as shown in the Western blot. GAPDH was used as loading control. C, HEP3B cells were transfected with a mixture of 2 siRNA sequences against LOXL4 (L4A an L4B), or both (L4) or the non-silencing control (Scr) for 72 hours before being harvested for protein for Western blot. The individual siRNA sequences as well as the pooled siRNA were able to knock down LOXL4 protein expression. GAPDH was used as loading control.

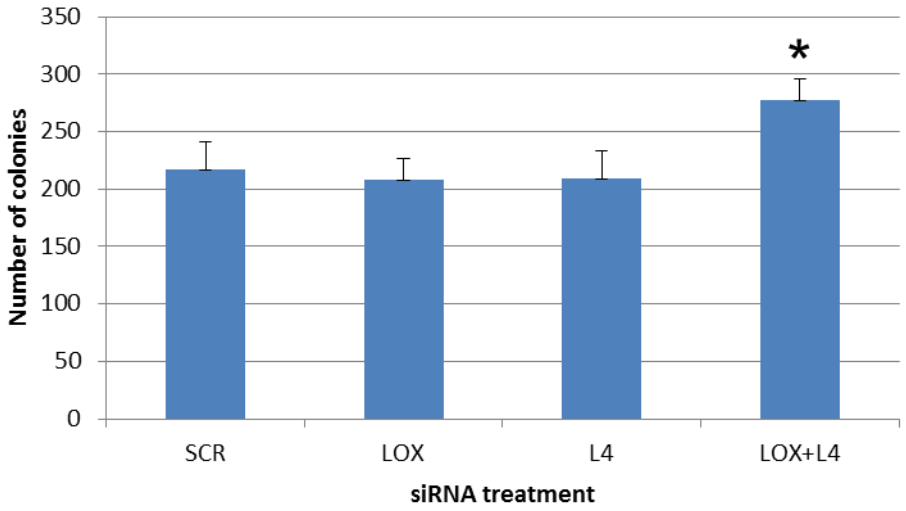


Figure 4.32 Effect of knocking down LOX or LOXL4 in HEP3B cells. Cells were transfected with LOX siRNA (LOX), or LOXL4 siRNA (L4), or both (LOX+L4) or non-silencing control siRNA (SCR). They were allowed to recover overnight before being counted and replated at low density. After 10 days, the colonies were stained with crystal violet and the wells were imaged and the number of colonies counted. The average number of colonies in each of the triplicate wells were plotted against the treatment. The error bars showed standard deviations. The knockdown of LOX or LOXL4 did not change the ability of the cells to form colonies. However, the knockdown of both LOX and LOXL4 actually increased the colony formation in these cells ($p=0.025$). Error bars showed standard deviations.

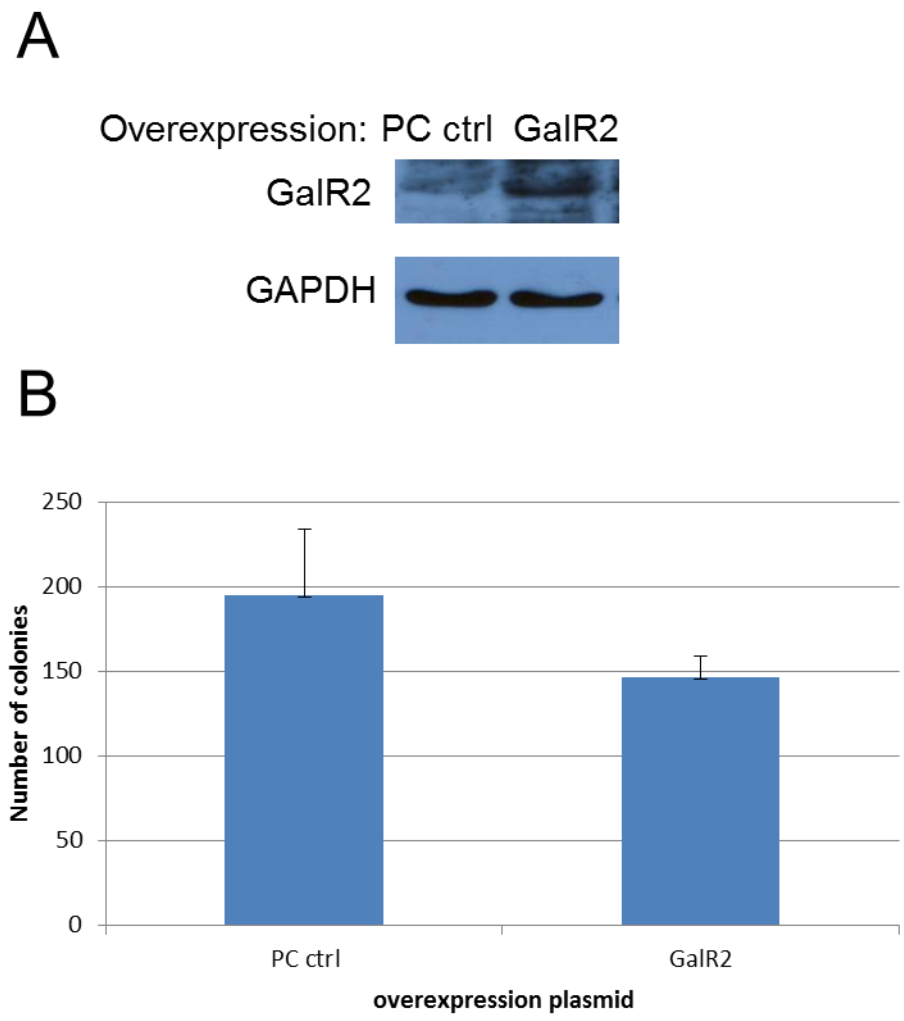


Figure 4.33 Effect of overexpressing GalR2 in HEP3B cells. Cells were transfected with pCMV-GalR2 plasmid (GalR2), or empty vector control (PC ctrl). They were allowed to recover overnight before being counted and replated. A, Western blot to show the overexpression of the GalR2 compared to the empty vector control (PC ctrl). B, Eight days after replating, the colonies were stained with crystal violet and the wells were imaged and the number of colonies counted.The average number of colonies in each of the triplicate wells were plotted against the treatment. There was slight reduction in the number of colonies after overexpression of GalR2, but it was not statistically significant ($p=0.11$). The error bars showed standard deviations.

CHAPTER 5

DISCUSSION

CHAPTER 5 DISCUSSION

5.1 Upregulation of HDAC1 and HDAC2 in hepatocellular carcinoma (HCC)

We examined expression of both HDAC1 and 2 in 28 pairs of HCC and their matched adjacent non-tumor tissues by Western blot and 179 pairs of HCC by tissue microarray. To minimize the effect of variation among patients, the expression of HDAC1 and 2 in each tumor was normalized to the matched adjacent non-tumor in the same patient as control. Our results consistently showed that both HDAC1 and HDAC2 were upregulated in the majority of the HCC samples compared to the matched adjacent non-tumor controls. The level of protein upregulation can be 7-fold or higher in some samples, as quantitated by densitometry after Western blot. Based on immunohistochemistry, localization of HDAC1 and 2 were found to be strongest in the nucleus, which is consistent with the known functions of class I HDACs as regulators of gene expressions.

Numerous studies have found HDAC1 and HDAC2 to be upregulated in other cancers. For example, HDAC1 and 2 were upregulated in renal cancer, prostate cancer, colorectal cancer, and gastric cancer (Fritzsche et al., 2008; Weichert et al., 2008b; Weichert et al., 2008c; Weichert et al., 2008d). In liver cancers, Rikimaru et al also reported the high expression of HDAC1 in HCC samples but they did not look at HDAC2 expression (Rikimaru et al., 2007). Another group found class II HDACs (HDAC4, 5, 6, 7, and 10) to be higher in HCC than in normal and cirrhotic livers, but they did not study the expression of class I HDACs (Bai et al., 2008).

Samples with downregulated HDAC1 and HDAC2 in our HCC tissues compared to the matched adjacent non-tumors were very few (3 out of 179 samples). However, there have been reports of the downregulation or loss of HDACs in cancer.

In endometrial adenocarcinoma, there was one report of fewer number of HDAC1-positive staining of the stromal and epithelial cells in the tumor tissues compared to the normal tissues (Krusche et al., 2007). For HDAC2, a frameshift mutation has been reported in colorectal cancer (Ropero et al., 2006). This mutation led to the loss of HDAC2 protein and was found in sporadic carcinomas with microsatellite instability as well as in tumors in people with hereditary nonpolyposis colorectal cancer syndrome.

While our results clearly showed upregulation of both HDAC1 and 2 in HCC tumor samples, they only reflected the protein expression level without demonstrating that the HDACs are enzymatically active. HDAC activity can be modulated by post-translation modification such as phosphorylation and sumoylation (David et al., 2002; Galasinski et al., 2002; Tsai and Seto, 2002). Therefore, it will be interesting to determine the acetylation status of histone H3 and H4 using specific antibodies in the clinical samples as a surrogate measure of HDAC activity in future studies.

5.2 Correlation between HDAC1 and HDAC2 expression with clinicopathological parameters

5.2.1 Patient survival

HDAC1 and 2 indices for each patient were obtained based on the level of upregulation of HDAC1 and HDAC2 in the tumor compared to the matched non-tumor samples. The patients were categorized into either high or low expression for HDAC1 and HDAC2 and the cumulative survival ratio of the population of samples was plotted over 6 years. Kaplan-Meier analysis showed that those with higher upregulation of HDAC1 have poorer survival than those with no or low upregulation. When multivariate analysis was done using cox regression, HDAC1 was the

independent factor for poor prognosis. On the other hand, there is no statistically significant difference for patients with high or no/low HDAC2 upregulation in terms of survival.

Various groups have studied the correlation of HDAC1 and 2 expressions with patient survival in different types of cancer. Of these, neither HDAC1 nor HDAC2 was found to have statistically significant effect on patient survival in cutaneous T cell lymphoma, ovarian serous carcinoma, and endometrial endometriod carcinoma (Marquard et al., 2008; Weichert et al., 2008a). On the other hand, HDAC1, but not HDAC2, was found to be a significant prognosis factor for patient survival in ovarian endometriod carcinoma (Weichert et al., 2008a); while HDAC2, but not HDAC1, was found to be significant in colorectal cancer and prostate cancer (Weichert et al., 2008c; Weichert et al., 2008d); and both HDAC1 and 2 were significant in gastric cancer (Weichert et al., 2008b). Table 5.1 summarized these findings.

Table 5.1 Significance of HDAC1 and HDAC2 expressions on patient survival in the various cancers. P-values< 0.05 were considered significant and were printed in bold.

Type of Cancer	HDAC	Significance	Reference
Cutaneous T-cell lymphoma (n=59)	HDAC1	P=0.3	(Marquard et al., 2008)
	HDAC2	P=0.94	
Ovarian serous carcinoma (n=176)	HDAC1	P=0.77	(Weichert et al., 2008a)
	HDAC2	P=0.97	
Endometrial endometriod carcinoma (n=123)	HDAC1	P=0.18	(Weichert et al., 2008a)
	HDAC2	P=0.87	
Ovarian endometriod carcinoma (n=114)	**HDAC1**	**P<0.01**	(Weichert et al., 2008a)
	HDAC2	P=0.87	
Colorectal carcinoma (n=140)	HDAC1	P=0.35	(Weichert et al., 2008d)
	HDAC2	**P=0.03**	
Prostate carcinoma (n=145)	HDAC1	P=0.81	(Weichert et al., 2008c)
	HDAC2	**P=0.02**	
Gastric carcinoma (n=123)	**HDAC1**	**P<0.01**	(Weichert et al., 2008b)
	HDAC2	**P=0.02**	

In HCC, Rikimaru et al. found that the survival rate in patients with high HDAC1 expression is lower (Rikimaru et al., 2007). This is consistent with our

findings. In addition, they also demonstrated that high HDAC1 expression also indicated higher occurrence of cancer cell invasion into the portal vein, poorer histological differentiation, and a more advanced TNM stage. These suggested a role for HDAC1 in tumor aggressiveness and cell differentiation. However, they did not report if they had examined the expression of HDAC2 in their study.

Using clinical patient samples with matched controls, our study had demonstrated that HDAC1 but not HDAC2 is a significant prognostic factor for survival in HCC.

5.2.2 Other parameters

Neither HDAC1 nor HDAC2 expression was found to be correlated with most of the other clinicopathological parameters examined, such as race, smoking history, group staging or fibrosis. However, higher HDAC1 expression was found to be associated with hepatitis B virus (HBV) status of the patient. A study by Yoo et al. showed that HBV x-protein (HBx) can induce expression of HDAC1 at the transcription level (Yoo et al., 2008). Also, the protein expression of HDAC1 was increased in the hepatocytes of HBx transgenic mice. In addition, HBx was also known to interact with HDAC1 to cause transcription repression of other genes (Shon et al., 2009). The HBx protein can recruit HDAC1 to the promoter of insulin-like growth factor binding protein 3 (IGFBP3) and repress its gene expression via Sp1. Together these show that the correlation between HBV and HDAC1 in our clinical samples has physiological relevance, and HDAC1 expression is important in the development of HCC.

There was also association between pre-operative treatment and downregulation of HDAC1 in the tumor samples. The pre-operative treatment in these cases was embolization to shrink the tumor before resection surgery. It is not clear

how this may reduce HDAC1 expression. It could be due to the reduction of blood supply to the tumor cells leading to reduction in certain factors that maybe required for HDAC1 expression. However, due to the small number of samples with downregulated HDAC1 (only 11 out of 179), it is not possible to draw any conclusion.

5.3 Knockdown of HDAC1 and HDAC2 in the cells

5.3.1 Compensatory effects observed in cells

The siRNAs designed to knockdown HDAC1 and HDAC2 were specific and effective. Real time reverse transcription polymerase chain reaction (RT-PCR) results showed that the knockdown of HDAC1 did not affect HDAC2 and vice versa at the RNA level. However, at the protein level, the knockdown of HDAC1 slightly increased HDAC2 and vice versa, as shown by the Western blots.

Other studies have also observed similar cross-regulation phenomenon. Montgomery et al. constructed conditional knockout mice with deletion of HDAC1 in the heart and did not observe any change in the RNA level of HDAC2 or any other HDACs (Montgomery et al., 2007). The RNA level of HDAC1 was also unchanged in their conditional knockout mice with deletion of HDAC2. However, they did not check the protein expressions. In another study, knocking out HDAC1 in mouse embryonic stem cells increased HDAC2 protein expression, although it was not able to compensate for the function of HDAC1 (Lagger et al., 2002). Furthermore, the restoration of HDAC1 in these mouse embryonic cells can bring the HDAC2 expression back down (Zupkovitz et al., 2006).

Taken together, it seems likely that HDAC1 and HDAC2 are directly or indirectly involved in the regulation of the protein expression of the other. Because of the conventional role of HDACs as transcriptional repressors, and HDAC1 has been

known to autoregulate its own expression transcriptionally (Schuettengruber et al., 2003), it is tempting to postulate that the cross-regulation of HDAC1 and 2 also occurs at the transcription level. However, we did not observe any change in the HDAC2 mRNA after knocking down HDAC1 and vice versa based on our RT-PCR results. Therefore, HDAC1 and HDAC2 may be exerting its effect on each other by directly or indirectly affecting the translation or the post-translational modifications of the other, since HDACs have been known to deacetylate proteins other than histones. The detailed mechanisms would require further investigation.

5.3.2 Compensatory effects not observed in clinical samples

On the other hand, this cross-regulation may be context dependent. In our clinical samples, only 2 out of 11 liver tumors with downregulation of HDAC1 showed a corresponding upregulation in HDAC2, and none of the 7 liver tumors with downregulation of HDAC2 showed a corresponding upregulation in HDAC1. Therefore, while this cross-regulation may be observed by manipulating the expression of HDAC1 and 2 under experimental conditions in cells, we have yet to observe it under physiological settings in our clinical samples.

5.4 Effects of knocking down HDAC1 and HDAC2

5.4.1 Reduction of colony formation and proliferation

In all of the 3 liver cancer cell lines (HEP3B, HEPG2, and PLC5) and 1 colon cancer cell line (HCT116) tested, the knockdown of both HDAC1 and 2 together were able to reduce the clonogenic potential of the cells. Unlike other assays that quantify cell numbers or amount of cell death, colony formation assay measures the ability of individual cells to form colonies when plated at low density. When both HDAC1 and 2 were knocked down, the cells failed to grow from single cells into colonies.

Growth of HEP3B cells was tracked over 6 days using the WST-1 assay which measures cell number based on their metabolic activity. The HDAC1+2 knockdown cells stopped proliferation between 72 to 96 hours post-transfection and the cell number began to decrease. From the Western blot data, the activation of caspase 3 and cleavage of its substrate PARP was observed from the 96 hours post-transfection. This time frame coincided with the WST-1 assay.

While the knockdown of HDAC1 and 2 can reduce cell growth in cancer cells, it did not affect cells that are non-proliferating, such as postmitotic cardiac cells, resting fibroblasts, non-dividing hepatocytes and B cells (Haberland et al., 2009; Wilting et al., 2010; Yamaguchi et al., 2010). For example, Haberland et al. showed that while the deletion of both HDAC1 and 2 has little effects on confluent primary fibroblasts and calvarial osteoblasts, it resulted in mitotic catastrophe in proliferating primary cells (Haberland et al., 2009). Similarly, in a study by Khabele et al., ovarian cancer cell lines (A2780, SKOV-3 and OVCAR3) were found to be more sensitive to HDAC inhibitor FK228-induced cell death than primary normal ovarian cells with a 3 to 5 folds difference in their half maximal inhibitory concentration (IC50) (Khabele et al., 2007). This differential effect of HDAC inhibition on cancer cells and non-proliferating cell makes HDAC inhibitors effective chemotherapy drugs.

5.4.2 Cell cycle profile showed increase in apoptosis

Cell cycle profiling of the HEP3B cells showed that when HDAC1 and 2 were knocked down together, there was increase in the percentage of cells in the sub-G1 phase, which is indicative of cells undergoing apoptosis characterized by fragmented DNA. There was consequently less cells in the G1 phase, but no change in the S phase or G2M phase.

Other studies also demonstrated increase in apoptosis after knocking down HDAC1 and 2. For example, conditional inactivation of HDAC1 and 2 in mouse embryonic fibroblasts (MEFs) resulted in increase in apoptosis (Yamaguchi et al., 2010). However, unlike our case, there was also G1 phase arrest. Another study which used MEFs also found senescence-like G1 cell cycle arrest in the double HDAC1 and 2 knockout cells, demonstrating the roles of these HDACs in controlling the G1 to S transition (Wilting et al., 2010).

Most HDAC inhibitors are known to induce cell cycle arrest at the G1 to S phase boundary, mediated by the retinoblastoma protein (pRb) and related proteins (Haggarty et al., 2003). HDAC inhibitors can induce *CDKN1A* which encodes for p21 protein that promotes hypophosphorylation of pRb, leading to cell cycle arrest (Richon et al., 2000). However, we did not observe this when we knockdown HDAC1 and 2 together in the HEP3B cells. Our gene profiling by microarray showed a 2.3-fold upregulation of *CDKN1A* mRNA after knocking down HDAC1 and 2 together, whereas the HDAC inhibitor PXD101 induced a 19.9 fold increase in the same cell line. Therefore, the slight induction of *CDKN1A* after knockdown of HDAC1 and 2 may not be sufficient to induce cell cycle arrest. Instead, apoptosis was observed.

Our data showed that there was activation of caspase 3 after knocking down HDAC1 and 2, as seen from the cleavage of the caspase 3 protein as well as the cleavage of its substrate PARP. While this clearly indicated the occurrence of apoptosis and the involvement of caspase 3, it cannot provide information on whether it was through the intrinsic or extrinsic pathway, or both. Our microarray data did not show any significant change in the expression of the various pro- or anti-apoptotic proteins in the mitochondrial (intrinsic) pathway or the molecules in the death-ligand

(extrinsic) pathway. Further studies can be done to study the apoptotic mechanisms involved.

5.4.3 Significant effects observed only when both HDAC1 and HDAC2 are knocked down together

For the colony formation assay, proliferation assay, and cell cycle profiling, the effects were observed only when both HDAC1 and 2 were knockdown together. Individual silencing of HDAC1 or HDAC2 did not show any significant effect. This could be due to HDAC1 and 2 having a compensatory effect on each other. In the absence of HDAC1, HDAC2 is able to compensate for the function of HDAC1, and vice versa. Therefore, knocking down just one of them did not have any significant effect on the cell. In fact, based on our Western blot data, knocking down one of them resulted in the increase of protein expression of the other. This further supports the hypothesis that such compensatory phenomenon occurs when only one of them was silenced. However, when both HDAC1 and 2 were knocked down together, the cell could no longer rely on the upregulation of either one of them to rescue the function of the other.

Numerous studies have demonstrated that the loss of either HDAC1 or HDAC2 alone did not affect the normal development of various tissues and cell types, such as the heart, glial cells, neurons, and B cells (Montgomery et al., 2007; Montgomery et al., 2009; Yamaguchi et al., 2010; Ye et al., 2009). It takes the inactivation of both HDAC1 and 2 to disrupt the proliferation and differentiation of various normal and tumorigenic cells.

On the other hand, there are also evidences for the unique functions of HDAC1 and 2 that cannot be compensated by the other. For example, the deletion of HDAC1, but not HDAC2, resulted in embryonic lethality in mouse due to

proliferation defects of the cells (Lagger et al., 2002). This demonstrated the critical role of HDAC1 in early embryogenesis, which cannot be substituted by HDAC2. In addition, when HDAC2 knockout mice were crossed with the tumor-prone APC (min) mice, there was reduction in the number of intestinal tumors (Zimmermann et al., 2007). Taken together, these data showed that while there is redundancy of HDAC1 and HDAC2 functions, there are also unique roles of HDAC1 and 2 under specific circumstances.

5.5 Role of enzyme activity in function of HDAC1 and 2

5.5.1 Synergistic reduction of HDAC activity after HDAC1 and 2 knockdown

HDAC activity assay was used to measure the global HDAC activity in HEP3B cells after knocking down HDAC1 and 2 individually and together. Our results showed that while the knockdown of HDAC1 alone partially reduced global HDAC activity, the knockdown of HDAC2 alone had no effect. When both HDAC1 and 2 were silenced together, there was synergistic reduction of global HDAC activity. Using the HCT116 p53-/- cells which do not produce endogenous HDAC2, we showed that the knockdown of HDAC1 alone resulted in a dramatic reduction of HDAC activity, similar to the fold change observed when both HDAC1 and 2 were knocked down together in cells that have endogenous HDAC1 and 2.

From our Western blot data described earlier, we observed a compensatory effect of HDAC1 and 2 on each other in terms of protein expression. This can explain why knocking down HDAC2 alone did not affect change in global HDAC activity. It is due to the compensatory upregulation of HDAC1. The knockdown of both HDAC1 and 2 eliminate such compensatory effect, and there was consequently a significant drop in the global HDAC activity. Nevertheless, we observed a partial reduction in

HDAC activity when HDAC1 was knockdown alone. This shows that the resultant upregulation of HDAC2 was not able to compensate for the reduction of HDAC activity after HDAC1 knockdown, possibly because HDAC1 is the major contributor to the cell's overall HDAC activity.

In addition, a series of *in vitro* studies demonstrated that HDAC1 and HDAC2 activities can be regulated by their own acetylation status. The deacetylase activity of HDAC1 was inhibited when it was acetylated by the HAT p300 (Qiu et al., 2006). Although HDAC2 cannot be acetylated by p300, its activity was inhibited by the acetylated HDAC1 (Luo et al., 2009). Moreover, the acetylated HDAC1 can indirectly inhibit activity of HDAC2 by heterodimerization. Each HDAC1/2 heterodimer requires 2 functional deacetylases to be active. Therefore, if one of them was inactive either by acetylation or inhibition of its catalytic domain, the heterodimer would be rendered non-functional as a deacetylase.

5.5.2 Effect of knocking down HDAC1 and 2 on colony formation is dependent on enzymatic activity

The HDAC1 and 2 wildtype and enzyme-dead mutant plasmids were constructed to test if the effect of knocking down HDAC1 and 2 on colony formation reduction can be rescued. Because HDAC2, both wildtype and mutant, cannot be overexpressed in cells that have endogenous HDAC2, only HDAC1 was overexpressed in the rescue experiment. Wildtype HDAC1, but not the enzyme-dead HDAC1 mutant, was able to partially but significantly rescue the reduction in colony formation after knockdown of both HDAC1 and 2. This rescue of the colony formation is attributed to the rescue of HDAC activity as evident from the HDAC activity assay, where the wildtype but not the enzyme-dead mutant HDAC1 plasmid can rescue HDAC activity in the presence of siRNA against HDAC1 and 2. In other

words, although we did not perform a full rescue of HDAC1 and 2, we were able to partially rescue the colony formation when we partially rescue the HDAC activity of the cell.

5.5.3 Protective effect of HDAC1 against PXD101-induced apoptosis

We used the HCT116 p53-/- cells to study the effect of overexpressing wildtype and mutant HDAC1 and 2, because we were unable to overexpress HDAC2 protein in other cell lines. This cell line has a mutation in the HDAC2 gene due to microsatellite instability (Figure 4.20). Therefore, it does not produce the full length HDAC2 protein endogenously, making overexpression possible.

The HDAC inhibitor PXD101 can induce apoptosis in HCT116 p53-/- cells in a dose-dependent manner. The overexpression of wildtype HDAC1 can rescue the cells from the PXD101-induced apoptosis. This protective effect is dependent on the HDAC activity as the enzyme-dead mutant of HDAC1 did not rescue the cells from apoptosis. Neither the HDAC2 wildtype nor mutant plasmid was able to exert such protective effect. This can be explained using the data from the HDAC activity assay, where only the overexpression of wildtype HDAC1, but not the mutant HDAC1, wildtype HDAC2, or mutant HDAC2, can increase the HDAC activity of the cells. Therefore, cancer cells with a higher expression of HDAC1 would be more resistant to HDAC inhibitors induced cell death.

It is interesting to note that while the overexpression of HDAC1 can increase HDAC activity in the cell, the overexpression of HDAC2 did not. This is consistent with our previous finding in another cell line where the knockdown of HDAC1 partially reduced HDAC activity but the knockdown of HDAC2 did not. This further supports HDAC1 as the major contributor to the cell's global HDAC activity.

In addition, the overexpression of mutant HDAC1 actually reduced the HDAC activity of the cells. This could be due to the overexpressed enzyme dead mutant competing with the endogenous wildtype HDAC1 to bind to substrates, thus reducing overall HDAC activity.

5.6 Apparent discrepancy between clinical samples and *in vitro* data

While our clinical data demonstrated that HDAC1 upregulation alone was correlated with lower patient survival, our *in vitro* studies showed that knocking down HDAC1 alone did not reduce tumor cell survival measured by colony formation and apoptosis in the HCC cells.

There could be several explanations for this apparent discrepancy. Firstly, HDAC1 may contribute to but not required in tumor cell survival and proliferation. During the process of carcinogenesis, a cell may gain survival and proliferation advantage by acquiring an upregulation in HDAC1. However, there may be other genetic and epigenetic events that occur concurrently, such that the removal of HDAC1 alone cannot kill the cell. Similar finding has been shown in a recent study of mitogen-activated protein kinase kinases (MEK), where MEK2 was found to be sufficient but not necessary for proliferation (Lee et al., 2011a). In that study, the knockdown of MEK2 alone has no effect on proliferation of the SK-MEL-28 melanoma cells. Both MEK1 and MEK2 must be silenced together to reduce cell proliferation. On the other hand, the reconstitution of MEK2 activity alone in the presence of a pan-MEK inhibitor was sufficient to drive proliferation of these cells, similar to our finding that HDAC1 overexpression was able to protect cells from PXD101-induced apoptosis.

Secondly, patient survival is influenced by many factors other than growth of the primary tumor alone. For example, metastatic potential of the tumor, the type of treatment and the resultant complications, many other factors that can affect the health of the patient can play important roles in determining how long the patient can live. Therefore, caution must be exercised when extrapolating *in vitro* data on tumor growth to the survival of the entire complex organism.

5.7 Genes regulated by HDAC1 and HDAC2

5.7.1 Comparing HDAC inhibitor PXD101 with knocking down HDAC1 and 2

Using microarray, we profile the gene expression in HEP3B cells after knocking down HDAC1 and 2 individually and together, as well as treating the cells with the HDAC inhibitor PXD101. Consistent with other studies, there were genes that were upregulated as well as genes that were downregulated after treatment despite the major roles of HDACs as transcription repressors. In comparison, the number of genes regulated at least 2 fold by PXD101 treatment was comparable to that by silencing HDAC1 and 2 together. The magnitude of fold change was also comparable between PXD101 treatment and HDAC1+2 knockdown. For example, among the upregulated genes, the median fold change was 1.6 fold for PXD101 treatment and 1.5 fold for HDAC1+2 knockdown. Therefore, the effectiveness of using the drug PXD101 as HDAC inhibitor to alter gene expressions seems to be similar to using HDAC1+2 knockdown.

5.7.2 Genes differentially regulated when both HDAC1 and 2 were knocked down together but not individually

In order to understand the mechanism by which HDAC1+2 knockdown affect cancer cell survival and proliferation, we focused on genes that were regulated after

knocking down both HDAC1 and 2 but not individually. From the microarray data, we identified 409 genes that were regulated at least 2 fold after knocking down both HDAC1 and 2, but not individually. These genes fall into a large collection of pathways with various functions. Some of these pathways have been implicated in cancer, such as the angiogenesis pathway and Wnt signaling pathway. Angiogenesis is the formation of new blood vessels, which is an important process needed for tumor growth and metastasis. The Wnt signaling pathway is important in organ development in many different species, but its aberrant activation is known to be associated with carcinogenesis (Barker et al., 2000).

5.7.3 Identification of possible mediators of the effect of HDAC1+2 knockdown on colony formation

We identified a list of genes that are similarly regulated by HDAC1+2 knockdown and PXD101 treatment, but are not regulated by knocking down HDAC1 or HDAC2 alone. Among these genes, we selected those with known functions in cancer cell survival and validated them using quantitative RT-PCR and Western blot. We further select 3 of these genes, lysyl oxidase (LOX), lysyl oxidase-like 4 (LOXL4), and galanin receptor 2 (GALR2), to test their effect on colony formation.

5.7.3.1 Lysyl oxidase (LOX) and lysyl oxidase-like 4 (LOXL4)

LOX and LOXL4 were found to be downregulated by HDAC1+2 knockdown and PXD101 treatment. The LOX family includes 5 members: LOX, LOXL, LOXL2, LOX3, and LOXL4. They share a C-terminal copper-binding domain and catalytic domain, but differ in their N-terminal domains which determine their individual roles (Kim et al., 2008).

LOX is a copper-containing amine oxidase that oxidize primary amine substrates to reactive aldehydes (Csiszar, 2001). It is important in the formation as

well as repair of the extracellular matrix by oxidizing lysine residues in elastin and collagen, stabilizing these fibrous proteins by initiating the formation of covalent crosslinkages (Kagan and Li, 2003). It has been shown to be involved in both tumor progression and tumor suppression, dependent on cellular location, cell type, and transformation status (Erler et al., 2006). For example, LOX was silenced by methylation in gastric cancer, and its re-introduction into the gastric cancer cell line MKN28 can reduce anchorage-independent cell growth (Kaneda et al., 2004). In addition, LOX was found to be downregulated in several oncogene-induced tumors and the knockdown of LOX can cause the transformation of rat fibroblasts (Giampuzzi et al., 2001). These evidences supported LOX as a putative tumor suppressor. On the other hand, breast cancer patients with high LOX expression in their tumors had poorer overall survival, and LOX was essential for hypoxia-induced metastasis (Erler et al., 2006). As such, the role of LOX in tumorigenesis and progression seems to remain unclear.

LOXL4 has an N-terminus that contains 4 scavenger receptor cysteine rich (SRCR) domains, which are usually found in cell-membrane associated proteins functioning in cell adhesion (Hohenester et al., 1999). It is overexpressed in head and neck squamous cell carcinoma (Holtmeier et al., 2003), but has also been demonstrated to be silenced in human bladder cancer (Wu et al., 2007). In the HCC cell line PLC5, LOXL4 was demonstrated to be a target gene of TGFbeta1 and can inhibit TGFbeta1-induced cell motility (Kim et al., 2008).

Because both LOX and LOXL4 was downregulated after HDAC 1+2 knockdown as well as after PXD101 treatment, we used siRNA to silence their expression to test if it affects colony formation. When LOX and LOXL4 was knocked down individually in HEP3B cells, there was no effect on colony formation. When

they were knocked down together, there was actually an increase in the colony formation. This shows that it is unlikely for these 2 genes to be mediators of the HDAC1+2 knockdown-induced effect on colony formation. However, this data is consistent with the findings in some studies described earlier, which showed tumor suppressor characteristics in LOX and LOXL4.

5.7.3.2 Galanin receptor 2 (GALR2)

GALR2 was upregulated by HDAC1+2 knockdown and PXD101 treatment. Galanin is a neuropeptide with a wide range of distribution and effects, mediated through the 3 G proteins-coupled receptor subtypes: galanin receptors 1 to 3 (GALR1-3). The expressions of galanin and its receptors are also found in some tumors such as glioblastoma, neuroblastoma, small cell lung cancer, and head and neck cancer (Berger et al., 2004; Berger et al., 2003; Wittau et al., 2000). Kanazawa and his team hypothesized GALR1 and GALR2 to be tumor suppressors and important therapeutic targets in head and neck squamous cell carcinoma (Kanazawa et al., 2010). They demonstrated that by overexpressing GALR1 in head and neck squamous cell carcinoma (HNSCC) cells that lack endogenous GALR1, galanin can suppress cell proliferation (Kanazawa et al., 2007), while GALR2 can induced caspase 3-dependent apoptosis (Kanazawa et al., 2009).

In our HCC model system, the knockdown of HDAC1 and 2, as well as treatment with PXD101, increased expression of GALR2 but did not change that of GALR1 and GALR3 which are very lowly expressed in the HEP3B cells. The expression of the galanin ligand was not changed after HDAC1+2 knockdown, but was increased after PXD101 treatment. We tested if the overexpression of GALR2 reduce colony formation in these cells using a pCMV-GALR2 plasmid we requested from Dr. Kanazawa's lab. Our results showed that GALR2 can decrease colony

formation, but it was not statistically significant. There are various possibilities, one of which could be that the overexpression of the receptor GALR2 alone, without the galanin ligand, was not enough to trigger the downstream effect. Although the upregulation of galanin mRNA was not detected after HDAC1+2 knockdown base on our microarray data, there could still be an increase in the release of the galanin peptide. This can be verified by performing an ELIZA of the cell culture media after HDAC1+2 knockdown, or by immunofluorescence using anti-galanin antibody to detect the ligand binding to the cell surface. If there is increase galanin secretion and binding on the cell surface, then we need to perform the overexpression experiment with the addition of the galanin peptide to test the effect of colony formation.

5.8 Future studies

5.8.1 HDAC and the Wnt signaling pathway in HCC

The differentially regulated genes after HDAC1+2 knockdown were mapped into many different pathways, one of which is the Wnt signaling pathway. The Wnt signaling pathway is important in normal liver physiology as well as pathology, by regulating differentiation, proliferation, and survival (Chiba et al., 2007; Thompson and Monga, 2007; Zeng et al., 2007). For example, activating mutation of Wnt signaling was found in 90% of colorectal cancers, though less frequently in HCC with a rate of about 18% (Giles et al., 2003). In addition, HDACs have been shown to epigenetically regulate genes in the Wnt signaling pathway. For example, the Wnt antagonist Dickkopf-1 (DKK1) was upregulated after treatment with HDAC inhibitor TSA in medulloblastoma cell line D283 (Vibhakar et al., 2007). Although we did not observe this upregulation from our data, we found the downregulation of the Wnt target genes, *CCND1* (encodes for cyclin D1), as well as the upregulation of *PRKCA*

(encodes for protein kinase C alpha) which is involved in the Wnt/calcium pathway. Future work can be done to test the hypothesis that HDAC1+2 knockdown induced reduction of colony formation is mediated via the Wnt signaling pathway. In addition, we can test if there is any synergism in killing HCC cells using HDAC inhibitors with Wnt antagonist or inhibitors.

5.8.2 Enzyme-independent functions of HDAC1 and HDAC2

While we have established the enzyme-dependent functions of HDAC1 and 2 in cancer cell survival, we have not addressed their enzyme-independent functions. From our microarray, there were genes that were regulated by HDAC1+2 knockdown but not the HDAC inhibitor PXD101. These are possible target genes of HDAC1 and 2 that are regulated by mechanisms independent of the deacetylase activity. The functions of these genes and the pathway to which they belong can provide clues to their cellular effects. In addition, we can use the enzyme-dead mutants of HDAC1 and 2 to test their functional effects on normal and cancer cells.

5.8.3 Regulation of HDAC2

It is interesting to note that although HDAC2 mRNA was increased dramatically after transfection of the HDAC2 plasmid, the protein cannot be overexpressed in cells which have endogenous HDAC2. Only in a cell line that has a mutation which resulted in a truncated protein did we manage to overexpress HDAC2. Previous studies have shown that HDAC2 can be degraded by proteosomal degradation, but we still failed to overexpress the HDAC2 protein even when proteosomal inhibitors such as MG132 and Lactacystin were used. It appears that there is translation or post-translational mechanisms that tightly regulate the expression of the HDAC2 but not the HDAC1 protein. Investigating this mechanism can provide deeper insights into the difference between the 2 highly similar HDACs.

CHAPTER 6

CONCLUSIONS

CHAPTER 6 CONCLUSIONS

Using clinical samples, we studied the expression of HDAC1 and 2 in hepatocellular carcinoma (HCC) and their correlation with clinicopathological parameters. We demonstrated that both HDAC1 and HDAC2 proteins were upregulated in HCC compared to the matched adjacent non-tumor tissue. The upregulation of HDAC1, but not HDAC2, was correlated with lower patient survival. While the knockdown of HDAC1 and 2 individually did not affect HCC cell survival, the knockdown of both proteins simultaneously led to reduction in cell proliferation and colony formation, as well as increase in apoptosis. This is attributed to the synergistic reduction of global HDAC activity in the cell. When we knocked down HDAC1 and 2 individually, we observed that they exert a compensatory effect on each other's protein expression, thereby preventing the drop in HDAC activity needed to induce cell death and reduction in colony formation and proliferation. The partial restoration of HDAC activity in the cell can partially rescue the cells from the reduction in colony formation induced by knockdown of HDAC1 and 2. Furthermore, the overexpression of the wildtype but not the enzyme-dead mutant of HDAC1 can protect the cells from HDAC inhibitor induced apoptosis. Drug developers are seeking more isoform-specific HDAC inhibitors in hope of reducing off-target effects when treating cancer patients with HDAC inhibitors. However, it is important to note that the functional effect of inducing cell death may be compromised when only single isoform is inactivated due to the cross regulation of the various isoforms and compensatory effects.

Using microarray, the gene expression profiles of the HCC cell line HEP3B was examined after knocking down HDAC1 or/and HDAC2, as well as after

treatment with the HDAC inhibitor PXD101. We found that the number of genes regulated and the magnitude of fold change was comparable between HDAC1+2 knockdown and PXD101 treatment. We focused on the group of genes that were regulated by HDAC1+2 knockdown and PXD101 treatment, but not by knockdown of HDAC1 or HDAC2 alone. We validated a number of genes using RT-PCR and western blot, and selected 3 of them for testing their involvement in cell survival using our system. However, none of them showed statistically significant effects on colony formation in the HEP3B cells.

When the HDAC1+2 knockdown regulated genes were grouped into the pathways to which they belong, we identified the Wnt signaling pathway as the one most significantly affected by the HDAC1+2 knockdown. This suggests that HDAC1 and 2 may exert their effect on cell survival and proliferation through the Wnt signaling pathway. Further work needs to be done to test this hypothesis.

In conclusion, we have demonstrated the significance of the upregulation of HDAC1 and 2 observed in hepatocellular carcinoma. Their contribution to the survival and proliferation of tumor cells is dependent on their enzymatic activity.

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APPENDICES

APPENDICES

General Reagents

dd water

Distilled deionized water was used in all experiments

Autoclaving

All autoclaving was carried out at 15lb/sq inch for 20min.

DEPC-treated water

1ml of DEPC was added per liter of dd water. Solution was shaken and left overnight at room temperature. To inactivate remaining DEPC, the solution was autoclaved.

1XPBS

To 800ml of dd water, add:

8g	Nacl
0.2g	KCl
0.24g	KH2PO4
1.44g	NaHPO4

Adjust pH to 7.0 with HCl and add dd water to 1 liter.

Reagents for DNA gel electrophoresis

1% agarose gel

Dissolve 0.5g DNase-free agarose in 50ml 0.5% TBE. Boil mixture in microwave oven. Allow to cool and add 1µl of 10mg/ml ethidium bromide solution.

10X Tris-Borate EDA (TBE) buffer, pH 8.2 (per liter)

0.89M	Tris base
0.89M	Boric acid
0.01mM	EDTA

Reagents used for transformation

LB agar medium (per liter)

To 950ml of water, add:

10g	Bacto-trypton
5g	Bacto-yeast extract
10g	NaCl
20g	Agar

Dissolve solutes. Adjust pH to 7.0 with 5N NaOH. Adjust volume to 1 liter with dd water. Sterilize by autoclaving. Cool to 50oC. Add antibiotics to desired concentration and pour onto petri dishes. Allow to solidify at room temperature and store at 4oC.

LB broth (per liter)

To 950ml of water, add:

10g	Bacto-trypton
5g	Bacto-yeast extract
10g	NaCl

Dissolve solutes. Adjust pH to 7.0 with 5N NaOH. Adjust volume to 1 liter with dd water. Sterilize by autoclaving. Store at 4oC.

Reagents for Western Blot

Lysis buffer for protein extraction

6M	Urea
1%	2-mercaptoethanol
50nM	Tris buffer pH 7.4
1%	SDS

Dissolve all solution in 1X PBS.

12% SDS-PAGE resolving gel (10ml)

4ml	30% Bis/Acrylamide
2.5ml	1.5 M Tris-HCl, pH 8.8
3.3ml	dd water
100µl	10% SDS
100µl	10% APS
4µl	Temed

4% SDS-PAGE stacking gel (4ml)

0.53ml	30% Bis/Acrylamide
0.49ml	1.5 M Tris-HCl, pH 6.8
2.86ml	dd water
40µl	10% SDS
40µl	10% APS
4µl	Temed

5X SDS/Glycine Buffer (per liter)

15.1g	Tris base
72g	Glycine
5g	SDS

5X sample loading buffer

10%	SDS
50%	Glycerol
7%	DTT
0.01%	bromophenol blue
50mM	Tris pH 6.8

Transfer buffer (per liter)

20%	ethanol
0.1%	SDS
14.4g	Glycine
3.03g	Tris

Reagents for HDAC assay

RIPA buffer

1%	NP40
1%	sodium deoxycholate
0.1%	SDS
0.15M	sodium chloride
50mM	Tris (pH 8.0)

Cell lysis buffer to lyse cell membrane

0.65M	sucrose
20mM	Tris (pH 8.0)
10mM	magnesium chloride
2%	Triton-X

NT buffer for nuclear extract

50mM	Tris (pH 7.4)
100mM	sodium chloride
5mM	magnesium chloride
5mM	calcium chloride
1%	NP40
1%	Triton-X
10U	DNase I

Add cocktail protease inhibitor (Roche) fresh before use.

Publications

Qiu GH, **Leung CH**, Yun T, Xie X, Laban M, Hooi SC. Recognition and Suppression of Transfected Plasmids by Protein ZNF511-PRAP1, a Potential Molecular Barrier to Transgene Expression. Molecular Therapy. 2011 May 3

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Leung CH, Wilson DA. Trans-neuronal regulation of cortical apoptosis in the adult rat olfactory system. Brain Research. 2003 Sep 12;984(1-2)182-8

Presentations at International Conferences

International Workshop on Cancer Stem Cell
10th-12th November 2005, Milan, Italy
Presentation: “Identification of side population in HCT116 and its metastatic derivatives’.

American Association for Cancer Research 96th Annual Meeting
16th-20th April 2005, Anaheim, California, USA
Presentation: “Inhibition of histone deacetylases 1 and 2 increases p21CIP1/WAF1 expression independent of p53 and Hsp90 in colon cancer cells”.

International Academy of Tumor Marker Oncology 21st Annual Meeting
21st-25th August 2004, Xi’an, China
Presentation: “Identification of splice variants of PRAP in colon and liver cancer”.

Association of Chemoreception Sciences 24th Annual Meeting
24th-28th April 2002 , Sarasota, Florida, USA
Presentation “Odor stimulation modulates apoptosis in olfactory cortex of the rat”.